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13. ABSTRACT (Maximum 200 Words)
Steroid hormones, estrogen and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivator proteins modulate the biological activity of these hormone receptors. We have cloned an E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP) as coactivators of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. We have examined this possibility by studying the expression patterns of E6-AP and estrogen receptor-alpha (ERα) in various human breast cancer cell lines and breast tumor biopsy samples. Additionally, we have correlated the expression profile of E6-AP with that of ER in breast tumor biopsies. To date, we have examined 13 samples of invasive breast cancer (IBC), 12 samples of ductal carcinoma in situ (DCIS) and a tissue array with36 different stages breast cancer samples by immunhistochemistry, and 19 samples by immunofluorescence. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. Furthermore, E6-AP is down regulated in invasive breast tumors compared with their adjacent normal tissues, whereas the downregulation of E6-AP was not seen in DCIS. The downregulation of E6-AP is stage-dependent. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. Presently, we are in the process of creating novel in vitro models in stable cell lines, which will overexpress E6-AP in a controllable manner.

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Introduction

Breast cancer is the most prevalent form of cancer (excluding skin cancer) among females in the United States (US). It is anticipated that one out of ten women will present with breast cancer at some point during her lifetime (1). The predominant treatment for patients with breast cancer is endocrine therapy, but these therapies are ineffective in some patients. Moreover, many patients, who initially respond to endocrine therapy, develop resistance later (2-6). Therefore, it is critical to identify the molecular mechanisms associated with breast cancer and with the development of endocrine-resistant tumors.

The steroid hormones, estrogen and progesterone, play a major role in the development of normal mammary gland and in breast tumor development (7-9). These molecules mediate their signaling through intracellular receptors called estrogen (ER) and progesterone (PR) receptors. ER and PR are members of a family of structurally related ligand-activated transcription factors (10, 11). These factors contain common structural motifs, which include a less well-conserved amino-terminal activation function (AF-1) that affects transcription efficiency, a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determines target gene specificity, and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2), the region, which mediates the hormone-dependent activation function of receptors (11). In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, they dissociate from cellular chaperones, dimerize with each other, phosphorylate, interact with coactivators, bind to the promoter region of the target gene, and subsequently recruite basal transcription factors to form a stable preinitiation complex (PIC). These steps are followed by up- or down-regulation of target gene expression (12).

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. A number of coactivators have been cloned to date, including SRC-family members (13, 14), TIF2 (GRIP1) (15-18), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (19-22), PGCs (23), SRA (24, 25), CBP (26, 27) and **E6-associated protein (E6-AP),** etc. and this list is still growing rapidly. Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (28, 29). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities, which contribute to their ability to enhance receptor-mediated transcription. SRC-1, p300/CBP, and RAC3/ACTR/AIB1 possess histone acetyl transferase activity (HAT) (19, 20, 30-32); E6-AP and RPF1/RSP5 contain ubiquitin-protein ligase activity (33, 34); and SUG1/TRIP1 contains ATPase activity (35). Ligand-activated receptors are thought to bring these activities to the promoter region of the target genes and presumably manifest part of their coactivation functions through these enzymatic activities. Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological response to steroids, vitamin D, and retinoids in different tissues. The

level of coactivator expression may contribute to variations in hormone responsiveness seen in the population and disruption in coactivator expression could lead to the pathologically hyper- or hypo-sensitivity to steroid hormones. The finding that disruption of the SRC-1 locus in mice resulted in an attenuated response to steroid hormones is consistent with this hypothesis (14).

Recently, our laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. **We have cloned an E3 ubiquitin-protein ligase, E6-AP, as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay (34). E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR).** E6-AP was previously identified as a protein of 100 kDa (36), present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (37). E3 enzymes have been proposed to play a major role in defining the substrate specificity of ubiquitin system (36, 38-41). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and E2 ubiquitin conjugating enzymes, UBCs. Firstly, ubiquitin is activated by UBA in an ATP-dependent manner, then the activated ubiquitin forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond (1, 42-45). In some cases, ubiquitin is transferred directly from E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (41). The carboxyl-terminal 350 amino acids (aa) of E6-AP contains a "*hect*" (homologous to the E6-AP carboxy terminus) domain, which is conserved among all E3 ubiquitin protein-ligases and E6-AP related proteins characterized to date (46) 48). The extreme carboxyl-terminal 100 aa of E6-AP contains the catalytic region, which transfers ubiquitin to the protein targeted for degradation. We have shown that the ubiquitin-ligase activity of E6-AP is not required for the coactivation function of E6-AP (34). It has been shown that the conserved cysteine (C) 833 residue in E6-AP forms a thioester bond with ubiquitin and is necessary for the transfer of ubiquitin to the proteins targeted for ubiquitination. The mutation of C833 to alanine (A) or serine (S) has been shown to eliminate the ubiquitin-protein ligase activity of E6-AP (41). **In cotransfection studies, we showed that an E6-AP bearing a C-to-S mutation at the critical site was still able to coactivate steroid hormone receptors.** These findings indicate that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity.

The role of E6-AP in mammary gland functions has not been studied yet. Considering, the influence of E6-AP as a coactivator on transactivation of target genes by ER and PR and also as an E3 ubiquitin-protein ligase, we are interested in studying the role of E6-AP in the development and progression of breast cancer. A large amount of evidence suggests that breast tumor development may involve coactivators of steroid hormone

receptors, especially those of ER and PR. It has been shown that altered expression of one nuclear receptor coactivator, AIB1, contributes to the development of hormone-dependent breast and ovarian cancers (19), while HER-2 *neu*(47) and Cyclin D are involved in breast cancer development(48, 49). Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function (19). Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways. It has also been shown that another steroid receptor coactivator, SRA is also elevated in breast tumors (25, 50). **Furthermore, we have recently shown that E6-AP is overexpressed in 90-95% of tumors using a mouse model of multistage mammary tumorigenesis developed by Medina et al (51, 52). Additionally, our data from human breast cancer biopsy samples shows that the majority of the advanced stage human breast tumors express high levels of E6-AP protein.** Since E6-AP is an E3 ubiquitin-protein ligase and recently, we have shown that ER is degraded through the ubiquitin-proteasome pathway (53), we also analyzed the expression profile of ER in human advanced stage breast cancers and compared it with that of E6-AP. **We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant.**

Body

In this original proposal, we hypothesized that the E3 ubiquitin-protein ligase, E6-AP, is an important modulator of the steroid hormone receptor-mediated signal transduction pathway, cell growth, and cell cycle control in the context of breast cancer development. In order to test this hypothesis we propose following objectives:

Aim 1. Expression analysis of endogenous E6-AP in human breast cancer samples and in human breast cancer cell lines, and comparison of the expression pattern of E6-AP with that of endogenous ER.
Aim 2. Generation of stably transfected breast cancer cell lines that overexpress wild-type and ubiquitin-protein ligase defective mutant E6-AP.
Aim 3. Analysis of the growth properties of stably transfected cell lines and in vivo analysis of tumorigenicity of these stably transfected cell lines in athymic nude mice.

Task 1. Expression analysis ER and E6-AP in different breast cancer cell lines.

Since we want to compare the expression profile of E6-AP with that of ERα, we performed dual fluorescent immunocytochemistry for MCF-7, T47-D, ZR75-1 and MDA-MB-231 cell lines. HeLa cell line was used as a negative control. Positive signal for ER is seen as green staining, whereas E6-AP is seen as red. As shown in Figure 1, ERα is expressed in MCF-7, T47D and ZR-75-1 breast cancer cell lines, which are known as ER positive, but it is negative in the HeLa cells and MDA-MB-231 cell line, which is known as ERα negative. The ER expression is nuclear in these cell lines. On the other hand, E6-AP is expressed in all the four breast cancer cell lines as well as in Hela cells. The E6-AP expression is both cytoplasmic and nuclear in MCF-7, ZR75-1 and MDA-MB-231 cell

lines. The MDA-MB-231 cell line expresses more E6-AP in nucleus than in the cytoplasm. The expression of E6-AP in T47-D cells is mainly nuclear. In this case HeLa cells were used as a positive control for E6-AP expression.

Task 2. Effect of steroids on the expression of E6-AP.

It is possible that steroid hormones (estrogens/progesterones) may regulate endogenous expression of E6-AP in breast cancer cell lines. To test this possibility, MCF-7, a hormone-dependent breast cancer cell line, was grown in the medium containing stripped serum for a week. Afterward, cells were grown either in the absence or presence of steroid hormones for 48 hours and the expression patterns of E6-AP were determined by fluorescent immunocytochemistry. Figure 2 suggests that the estrogen treatment have no significant effect on the expression of E6-AP. The E6-AP expression levels are identical both in the presence and absence of hormone. This data suggests that E6-AP regulation is not under the control of steroids.

As a control for these experiments, we also analyzed the effect of estrogen on the expression of PR and ER. It has been established that estrogen upregulates the expression of PR protein and it downregulates the levels of ER in MCF-7 cells (54). As expected, Figure 3 demonstrated that estrogen treatment increases the expression of PR protein. In contrast, estrogen down regulates ER expression.

Task 3. Expression analysis of E6-AP and ER in breast tumor samples.

As mentioned above, the ubiquitin pathway enzyme, E6-AP acts as a coactivator of steroid hormone receptors. Furthermore, we have also demonstrated that the ER protein, which is a major modulator of normal mammary gland development and breast tumor development, is rapidly degraded in mammalian cells in an estrogen-dependent manner via the ubiquitin-proteasome pathway. Additionally, our *in vitro* studies suggest that ER degradation observed in mammalian cells is dependent on the ubiquitin-proteasome pathway (53). Besides, Western blot analysis of advanced stage human breast cancer samples found varied levels of expression of E6-AP and an inverse correlation between the expression of E6-AP with that of ER. To further explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer, we analyzed immunohistochemically the expression of E6-AP in invasive breast cancer (IBC) and ductal carcinoma in situ (DCIS) samples and compared their expression with their adjacent normal breast tissues. We also analyzed the expression levels of E6-AP in different stages breast cancer samples by immunohistochemistry. To compare the expression of E6-AP with that of ERα, dual immunofluorescent staining was applied.

A. Immunohistochemistry

In order to study the expression profile of E6-AP in breast tumors and in normal breast tissues, we performed immunohistochemical analysis. Figure 4 shows a representative breast cancer case. The normal tissue, DCIS and IBC were all found in one slide, making them ideal controls for each other. In normal human breast tissues, E6-AP is highly

expressed in the cytoplasm of the ductal epithelial cells. Compared with the normal tissue, the immunostaining of E6-AP is greatly decreased in the IBC, whereas there was no significant change of E6-AP expression in the DCIS. We analyzed 13 cases of IBC and 12 cases of DCIS with their adjacent normal tissues. To compare the expression of E6-AP in tumors with that in normal tissues, the immunostaining results were evaluated using automated cellular imaging system (ACIS, Chroma Vision Medical Systems, Inc., San Juan Capistrano, CA). This system combines color based imaging technology with automated microscopy to provide quantitative information on intensity of staining (and if desired the percent of positively stained cells). **Figure 5** summarizes the scanning results of E6-AP immunostainig in IBC and their adjacent normal tissues. "**A**" is a table showing the intensity of E6-AP immunostaining in both IBC and normal tissues from each of the 13 breast cancer cases. "**B**" is a bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side. All of the 13 IBC samples express reduced level of E6-AP compared with their adjacent normal tissues. In average, there is a 25% decrease of E6-AP expression in tumor than in the normal tissues. Student paired *t*-test indicates that the difference is statistically significant (p=0.000001). **Figure 6** summarizes the scanning results of E6-AP immunosaining in DCIS and their adjacent normal tissues. "**A**" is a table showing the intensity of E6-AP immunostaining in both IDCIS and normal tissues from each of the 12 breast cancer cases. "**B**" is a bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side. Unlike in IBC, the expression levels of E6-AP is not significantly different from that of their normal tissues (p=0.9397). Taken together, these results indicate that the downregulation of E6-AP in breast cancers is a relatively late event.

Next, we performed immunohistochemical staining of E6-AP on a microarray breast cancer slide which contains 36 different stages breast cancer samples. As shown in **Figure 7**, the immunostaining of E6-AP was graded manually according to the overall density of brown color in each sample, with the highest level as 4, the lowest as 0 and the intermediate as 1, 2 and 3. In comparison to the normal breast tissues, the levels of E6-AP in cancers were mostly downregulated. However, E6-AP expression goes down at Stage I, and then goes up a bit at Stage IIA, after that, its expression goes down again. To analyze the differences between different stages of breast cancers, Wilcoxon rank-sum test was used, as shown in **Figure 8**. Combined with Table 1, it is observed that the expression of E6-AP is decreased gradually from stage I to stage IIB, which is the lowest point, suggesting a possible role of E6-AP in the progression of breast tumors. These results suggested that the expression of E6-AP is stage-dependent and the changes of its expression levels might be involved in the progression of breast carcinomas.

B. Immunofluorescence

Combining the data from Western blot and Immunohistochemistry analysis, it is suggested that E6-AP is down regulated in breast tumors and the expression of E6-AP is correlated with that of ER alpha. To confirm this, we further performed dual color immunofluorescence to analyze the expression of E6-AP with that of ER. As shown in Figure 9, E6-AP and ER is differently expressed in tumors and in normal tissues: (1) ER is expressed in the nucleus, whereas E6-AP is expressed in the cytoplasm; (2) In normal

tissues, ER is discontinuously expressed in the epithelial cells, whereas E6-AP is ubiquitiously expressed in the epithelial cells; (3) In tumor tissues, ER is highly and ubiquitiously expressed in the epithelial cells, whereas the expression of E6-AP is low. Negative control was included in the experiment by omitting the primary antibody. This result further indicated that the inverse correlation of E6-AP with ER in breast tumors does exist. Nineteen human breast cancer samples were analyzed by dual immunofluorescence using antibodies aganist E6-AP and ER. The expression levels of E6-AP and ER were artificially graded, which is shown in **Figure 10**. Wilcoxon Rank Correlation Coefficient is 0.503, $p<0.05$, indicating an inverse correlation between the expression of E6-AP and ER.

Task 4. Generation of the expression plasmids for overexpression of E6-AP.

One of the goals of this proposal is to construct expression plasmids for either wild-type E6-AP or ubiquitin-protein ligase defective, C833S mutant E6-AP in order to make stable cell lines, which will overexpress wild-type or C833S mutant E6-AP proteins in breast cancer cell lines. As suggested in the proposal, we first cloned the relevant cDNAs of wild-type or mutant E6-AP into the mammalian expression vector pcDNA3.1 and attempted several times in transfecting the plasmids into MCF-7 cells. However, the transfection was not successful. We therefore switched to a regulable gene expression system by using Tet-Off-IN system (Clontech Laboratories). This system is composed of two critical components. The first component of the system is the **regulatory protein,** based on TetR (Tet repressor protein). In the pRevTet-Off-IN System, the amino acids 1–207 of TetR and the C-terminal 127 a.a. of the Herpes simplex virus VP16 activation domain were fused, forming a 37-kDa fusion protein. Addition of the VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). tTA is encoded by the pRevTet-Off-In regulator plasmid (**Figure 11**), which also includes a neomycin-resistance gene to permit selection of stably transfected cells. The second component is the **response plasmid (Figure 11),** which expresses a **gene of interest (Gene X)** under control of the tetracycline-response element, or TRE. The TRE, which consists of seven direct repeats of a 42-bp sequence containing the tetO (tet operator sequences), located just upstream of the minimal CMV promoter (P_{minCMV}), which lacks the strong enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is extremely low background expression of Gene X from the TRE in the absence of binding by the TetR domain of tTA. When cells contain both the regulatory (pRevTet-Off-In) and the response (pRevTRE) vectors, Gene X is only expressed upon binding of the tTA protein to the TRE (**Figure 12**). In the Tet-Off system, tTA binds the TRE and activates transcription in the absence of Tc (Tetracycline) or Dox (Doxycyclin). Therefore, the transcription of Gene X is turned on or off in response to Dox in a precise and dose-dependent manner.

One possible reason that we were not successful in generating stable cell lines using pcDNA3.1 vector is that constant overexpression of E6-AP is harmful to the cells that those cells which were transfected could not survive eventually. The Tet-Off-In system

allows us to turn on and off the transcription of our genes according to our needs, therefore, it is a better expression system than the previous one. We have cloned the wild-type and C833S mutant E6-AP cDNAs into the multiple cloning site of pRevTRE response plasmid. The E6-APs were tagged with four repeats of flag sequences at the N-terminal, therefore the recombinant proteins can be easily detected with an anti-flag antibody. We have performed transient transfection of both regulator and response plasmids into Hela cells in order to confirm whether the system works properly. Hela cells were maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-four hours before transfection, $1x10^6$ cells were plated in three 10 cm Falcon dishes. Cells in two of the three dishes were transfected with 5 μg of pRevTet-Off-In and pRevTRE/E6AP-WT by using Fugene 6 transfection reagent (Roche). The third dish of cells was untransfected and used as a control. Dox was added to one of the two transfected dishes 4 hrs later. After 24 hr, cells were harvested and lysed and equal amounts of proteins were resolved by a 10% SDS-PAGE then transfered to nitrocellulose membrane. The expression of recombinant E6-AP was detected using an anti-Flag antibody. As shown in **Figure 12**, in the untransfected Hela cells, no Flag was detected (-). When both pRevTet-Off-IN and pRevTRE/E6AP were introduced to the cells, a very strong signal of Flag was detected in the absence of Dox (-Dox), which represents the expression of recombinant E6-AP protein. The expression of this protein was inhibited when Dox was added to the culture (+Dox). This result indicated that the constructed plasmids were working as expected and we can move on to the next steps. We are currently in the process of making single stable cell line with regulator plasmid, pRevTet-Off-IN, into MCF-7 cells. Once the single stable cell line is established, we will then introduce the response plasmid, pRevTRE/E6AP(WT) or pRevTRE/E6AP(Mu), to make double stable cell lines. The stable expression of wild-type and C833S mutant E6-AP proteins will be achieved by initial transfection by electroporation and subsequent selection and propagation of clonal cells exhibiting antibiotic (Hygromycin and Neomycin) resistance. Then the expression patterns of transfected cell lines will be compared with untransfected cells using Western blot analysis. The stable clones that overexpress wild-type and C833S mutant E6-AP proteins at high levels will be selected and characterized further. To assess the functions of stably transfected wild-type and C833S mutant E6-AP forms, the transfected and the untransfected cells will be compared in their ability to activate estrogen-responsive reporter genes both in the absence and presence of estradiol.

Statement of work accomplished/in progress

Task 1. Expression analysis of ER and E6-AP in different breast cancer cell lines. **Accomplished.**

Task 2. Effect of steroids on the expression of E6-AP. **Accomplished.**

Task 3. Expression analysis of ER-alpha and E6-AP in breast tumor samples. **Accomplished.**

Task 4. Generation of the expression plasmids for overexpression E6-AP. **Accomplished.**

Task 5. Development of stable cell lines. **In progress.**

Task 6. Characterization of stable cell lines. **Not Attempted Yet.**

Task 7. Determination of growth properties of stable cell lines. **Not Attempted Yet.**

Task 8. Determine the tumorigenicity of stably transfected cell lines in athymic nude

mice. **Not Attempted Yet.**

Key Research Accomplishments

- Expression analysis of ER and E6-AP in different breast cancer cell lines has been completed.
- Effect of steroids on the expression of UbcH7 and E6-AP has been studied.
- Expression of ER and E6-AP has been analyzed.
- Expression profile of E6-AP has been compared with that of ER expression.
- Generation of the expression plasmids for overexpression of UbcH7 and E6-AP has been completed.
- Development of stable cell lines is in progress.

Reportable Outcomes

1. An article regarding the roles of coactivators, including E6-AP, in cancers, has been published in Molecular Cancer in November, 2002 (see appendix 2).
2. A poster entitled 'E6-associated protein, E6-AP is involved in the carcinogenesis of human breast and prostate' was posted at the 95th AACR annual meeting (Orlando, Florida, 2004).
3. An article entitled 'E6-associated protein, E6-AP, is involved in the carcinogenesis of mammary gland and prostate gland' has been submitted to Cancer Research.

Conclusions

We have successfully analyzed the expression of E6-AP and ER in different breast cancer cell lines. Additionally, we have also examined the effects of steroids on the expression profile of E6-AP and ER. In order to study the expression profile of E6-AP and ER in human breast tumors, we have examined 13 samples of invasive breast cancer (IBC), 12 samples of ductal carcinoma in situ (DCIS) and a tissue array of 36 breast cancer samples by immunhistochemistry, and 19 samples of invasive breast carcinoma by immunofluorescence. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. Furthermore, E6-AP is down regulated in IBC compared with their adjacent normal tissues, whereas the downregulation of E6-AP was not seen in DCIS. The downregulation of E6-AP in breast cancers is stage-dependent. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis. Presently, we are in the process of creating novel in vitro models in stable cell lines, which will overexpress E6-AP in a controllable manner.

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Appendices

1. Figures 1-12
2. Article published in Molecular Cancer
3. Article submitted to Cancer Research

Appendix 1

Figures 1-12

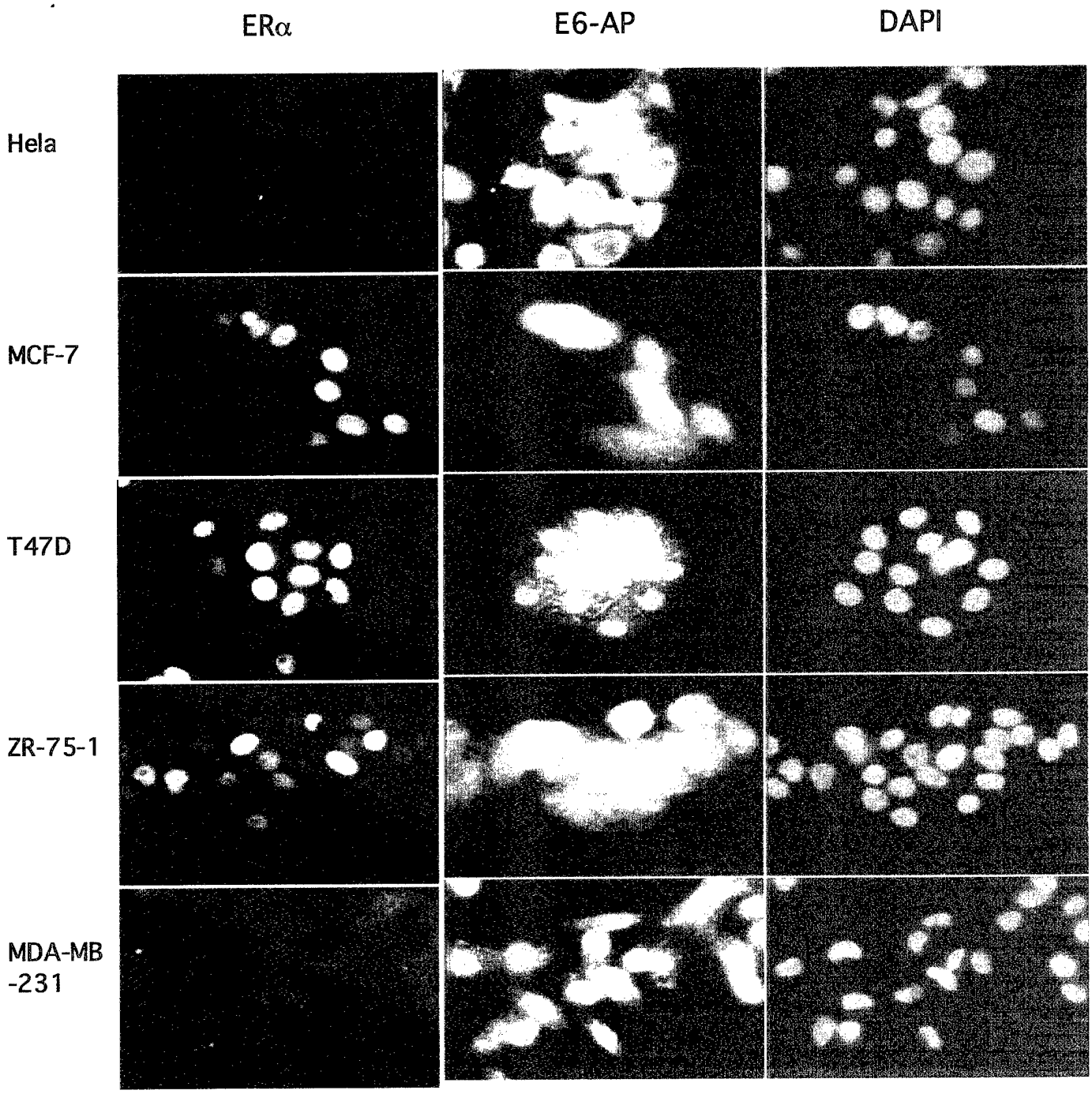


Figure 1: Expression analysis of ERα and E6-AP in different cell lines (Hela, MCF-7, T47D, ZR-75-1, and MDA-MB-231). Cells were grown on a chamber slide for 24 hours and expression of ERα and E6-AP was analysed by dual fluorescent immunocytochemistry using an anti-ERα antibody (6F11 from Novacastra) and an antibody against E6-AP. Positive signal for ER is seen as green spot and positive signal for E6-AP is seen as red spot. DAPI staining was used to show the localization of nucleus. ERα, ERα expression profile; E6-AP, E6-AP expression profile; DAPI, DAPI staining for nucleus.

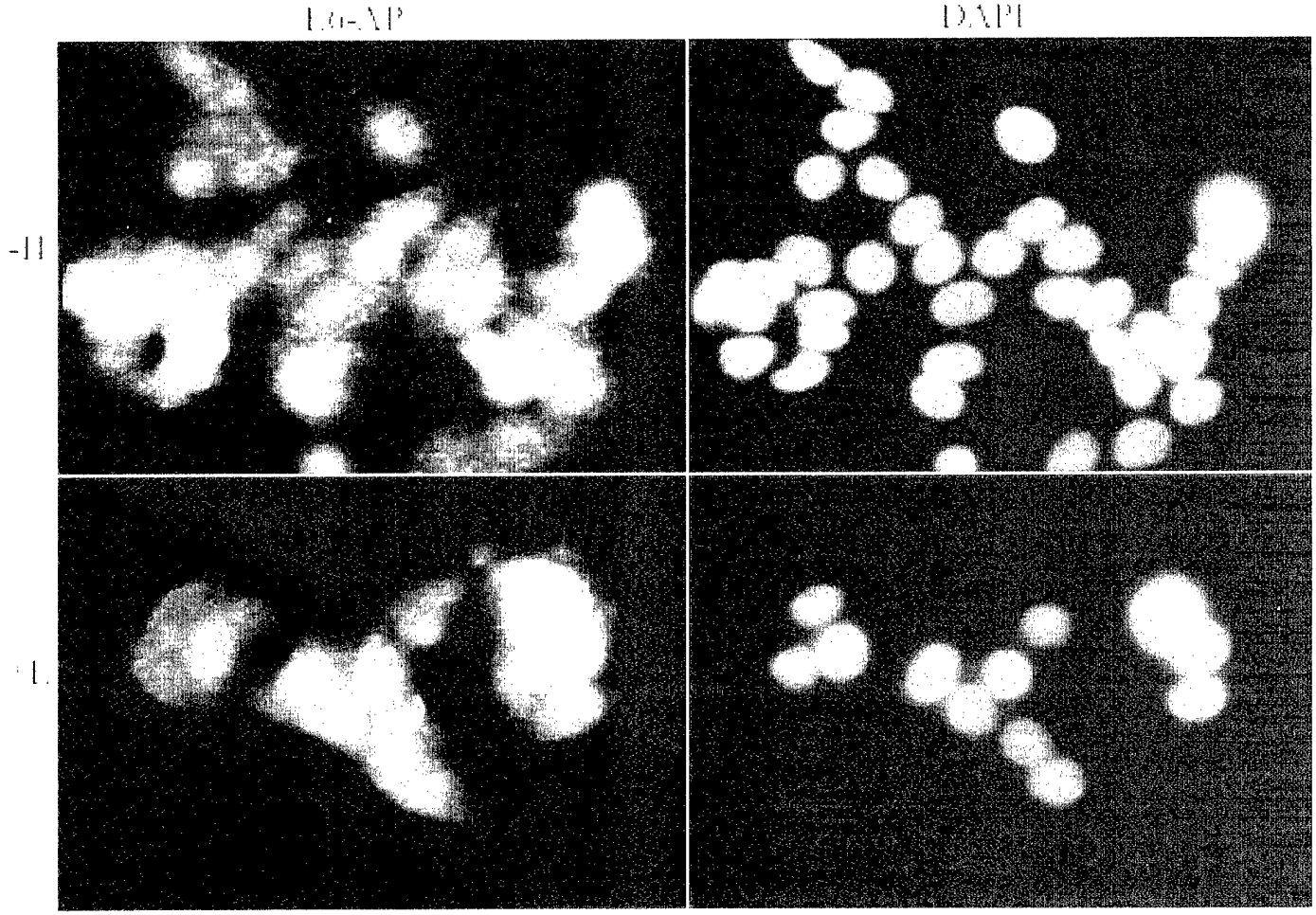


Figure 2: Effect of estrogen on the expression of E6-AP in MCF7 cells. Cells were grown on a chamber slide either in the absence (-E) or in the presence of estradiol (+E). 24 hrs after hormone treatment, the expression of endogenous E6-AP was analyzed by fluorescent immunocytochemistry using an anti-E6-AP antibody. Positive signal for E6-AP is seen as (red) spots and nucleus is seen as (blue) spots in DAPI staining. E6-AP: E6-AP expression profile; DAPI: DAPI staining for nucleus.

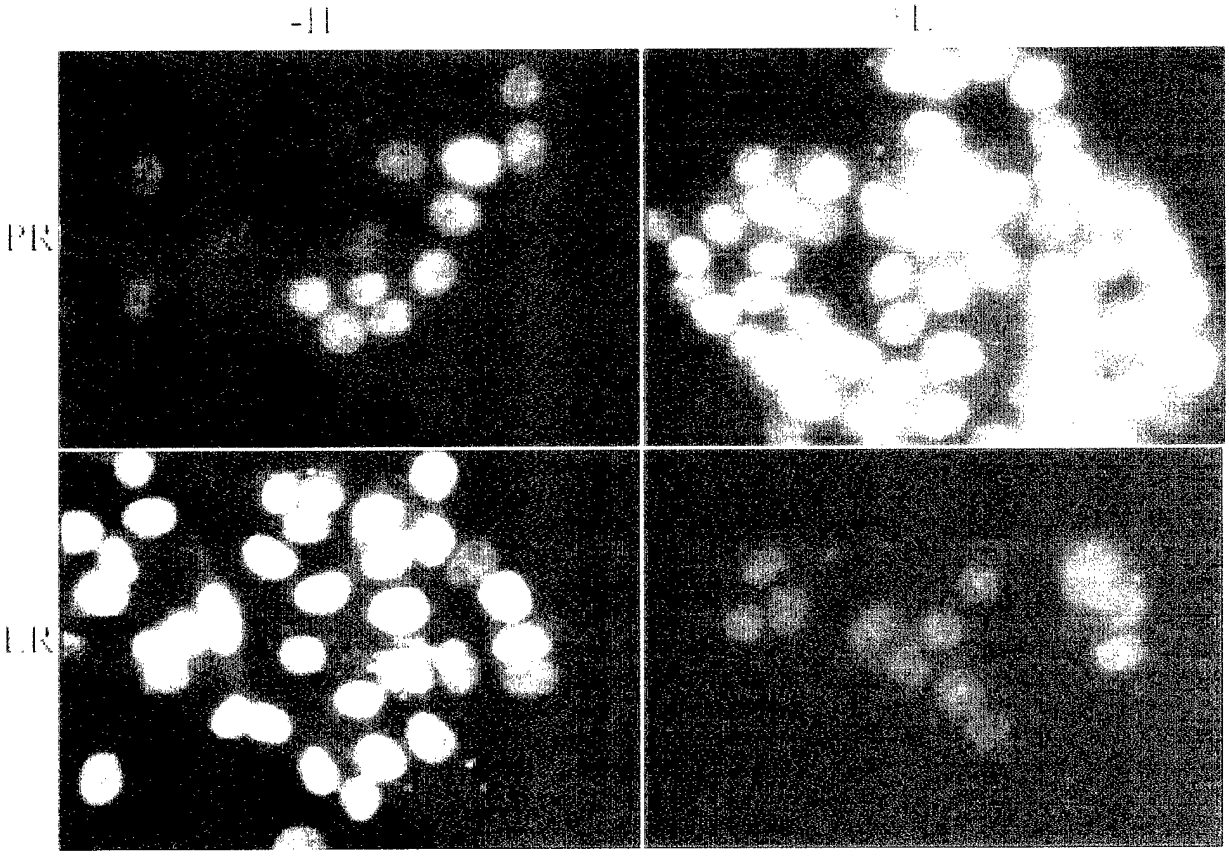


Figure 3. Effects of estrogen on the expression of PR and ERα in MCF-7 cells. Cells were grown on a chamber slide either in the absence (-H) or in the presence of estrodial (+E). 24 hrs after hormone treatment, the expression of endogeneous PR and ERα was analyzed immunofluorescence using anti-PR and ERα antibodies. Positive signal for PR and ERα is seen as green spots. PR, PR expression profile; ER, ERα expression profile.

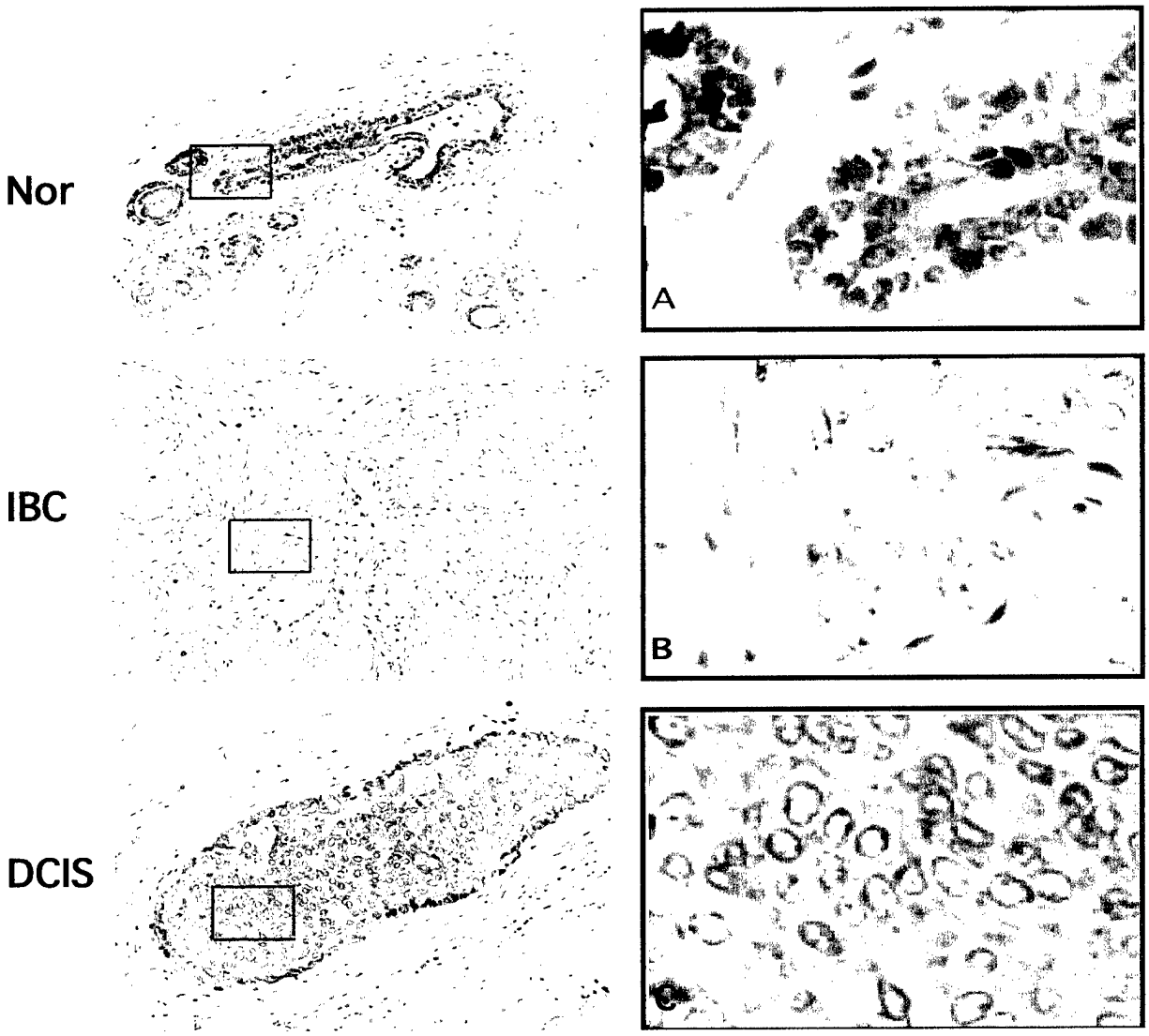


Figure 4: Immunohistochemical analysis of E6-AP in normal and malignant breast tissues-a representative case that include normal area, DCIS and IBC in one section. Paraffin-embedded human breast cancer biopsy samples were sectioned, deparaffinized, blocked, and incubated with an anti-E6-AP antibody. This was followed by incubation in a biotinylated anti-rabbit IgG and then the Vectastain ABC reagent (Vector Laboratories, Inc.). DAB kit (Vector Laboratories Inc.) was used to detect the bound antibody. After countersaining with Hematoxylin, the slides were dehydrated and mounted. Positive signal for E6-AP is seen as brown staining. Blue spots indicate the negative stained nuclei. a. Nor, normal area; b. IBC, invasive breast carcinoma; c. DCIS, ductal carcinoa in situ. "A", "B", and "C" are the enlarged image of "a", "b" and "c", respectively.

A. Immunostaining Intensity in DCIS and Normal Breast Tissues

Patient	Normal	IBC	T/N (%)
1	131	124	94.66
2	162	121	74.69
3	161	114	70.80
4	129	112	86.82
5	137	86	62.77
6	130	79	60.77
7	135	103	76.30
8	120	99	82.50
9	129	103	79.84
10	131	101	77.10
11	143	108	75.52
12	154	103	66.88
13	139	104	74.82
Average	138.54	104.38	75.34

(paired student *t*-test, p=0.000001)

B.

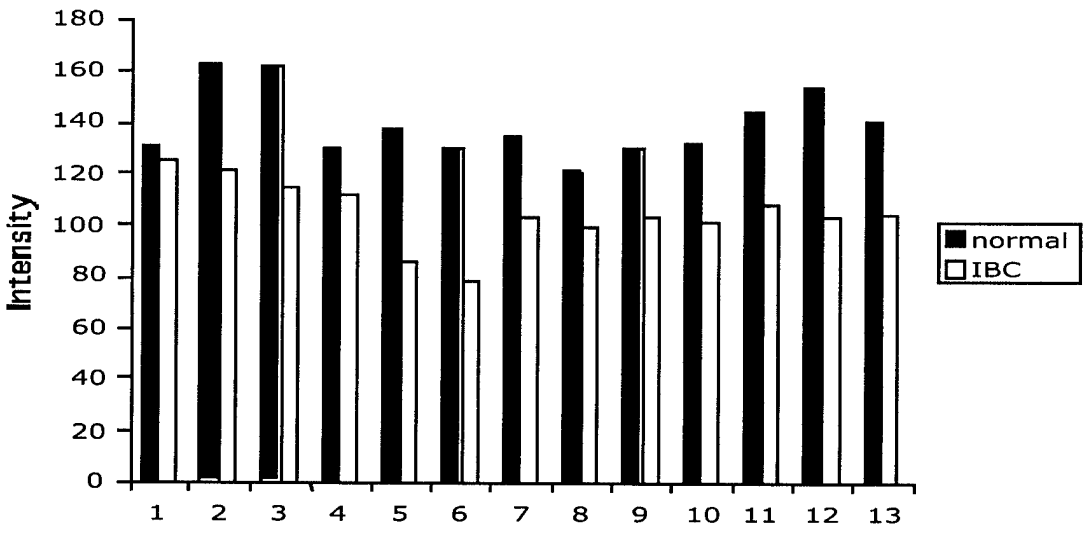


Figure 5: Intensity (mean brown value) of E6-AP immunostaining in normal and malignant human breast tissues. Thirteen paraffin-embedded human breast cancer biopsy samples including adjacent normal tissues were analyzed by Immunohistochemistry as mentioned above. The immunostaining results were evaluated using automated cellular imaging system. **A.** A table showing the intensity of E6-AP immunostaining in both normal and tumor tissues from each of the 13 breast cancer cases. **B.** A bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side.

A. Immunostaining Intensity in DCIS and Normal Breast Tissues

Sample No.	Normal	DCIS	T/N (%)
1	116	127	91.3
2	114	118	103.5
3	111	108	97.3
4	102	124	121.6
5	112	89	79.5
6	130	144	110.8
7	98	86	87.7
8	118	120	101.7
9	140	118	84.3
10	126	110	87.3
11	118	131	111.0
12	96	102	106.2
Average	115.08	114.75	98.52

(paired student *t*-test, p=0.9397)

B.

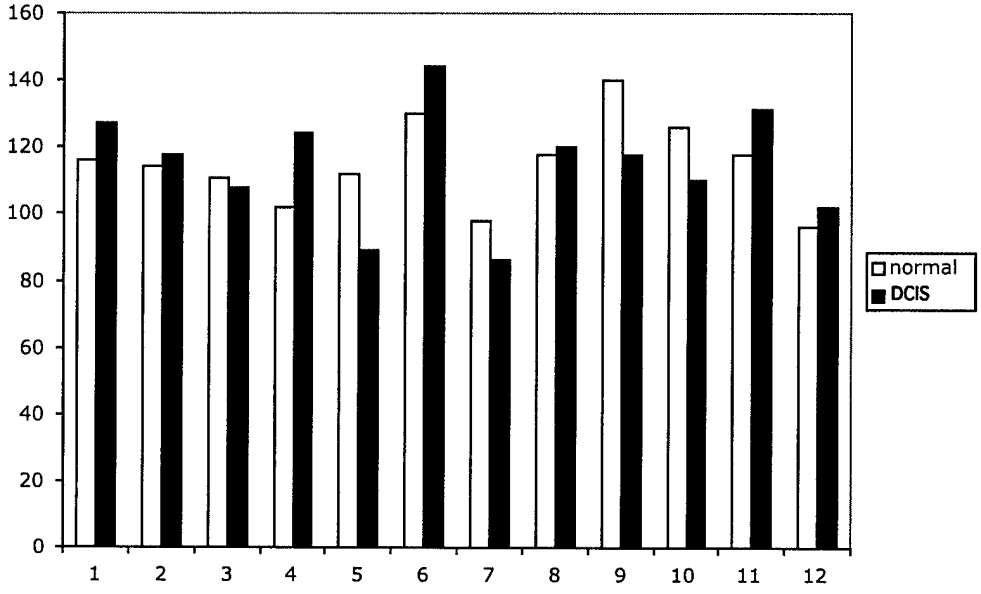


Figure 6: Intensity (mean brown value) of E6-AP immunostaining in DCIS and normal breast tissues adjacent to the tumors. Twelve paraffin-embedded human DCIS biopsy samples including adjacent normal tissues were analyzed by Immunohistochemistry as mentioned above. The immunostaining results were evaluated using automated cellular imaging system. **A.** A table showing the intensity of E6-AP immunostaining in both normal and tumor tissues from each of the 12 breast cancer cases. **B.** A bar chart comparing the intensity of E6-AP immunostaining in normal and tumor tissues side by side.

The Expression of E6-AP in Different Stages of Human Breast Cancers

Stage I	Stage IIA	Stage IIB	Stage IIIA	Stage IIIB
1	0.5	0.5	1.5	
1.5	1	1	2	
1.5	1	1	2	
2	1	1	2	
2	1.5	1	2	
2	1.5	1.5	2.5	
2	2	2		
2.5	2			
3	2			
	2			
	2			
	2.5			
	3			
	3			
N1=9	N2=14	N3=7	N4=6	
$\bar{X}$1=1.94	$\bar{X}$2=1.78	$\bar{X}$3=1.14	$\bar{X}$4=2.00	

Figure 7. Expression analysis of E6-AP in different stages of breast cancers. A tumor array with 36 breast cancer samples including tumors from stage I to stage IIIA in one slide was used for this study. Immunohistochemistry was carried out as mentioned above. The levels of expression were graded manually according to the overall density of brown color in each sample, with the highest level as 4, the lowest as 0 and the intermediate as 1, 2 and 3.

Comparison of The Expression Level of E6-AP between Different Stages of Breast Cancers

(Wilcoxon rank-sum test)

Stages Compared	Rank-sum T	n1,n2-n1	*P* value
I & IIB	32	7, 2	<0.01 *
IIA & IIB	52.5	7, 7	>0.05
IIIA & IIB	59.5	6, 1	<0.01 *
I & IIA	115	9, 5	>0.1
I & IIIA	50.5	6, 3	>0.1

Figure 8. Comparison of the levels of E6-AP in different stages of breast cancers. The immunostaining result of the tissue array slide, as mentioned in Figure 7, was statistically analyzed using Wilcoxon rank-sum test. The differences between different stages was compared. Combined with Figure 7, it is observed that the expression of E6-AP is decreased gradually from stage I to stage IIB, which is the lowest point, suggesting a possible role of E6-AP in the progression of breast tumors.

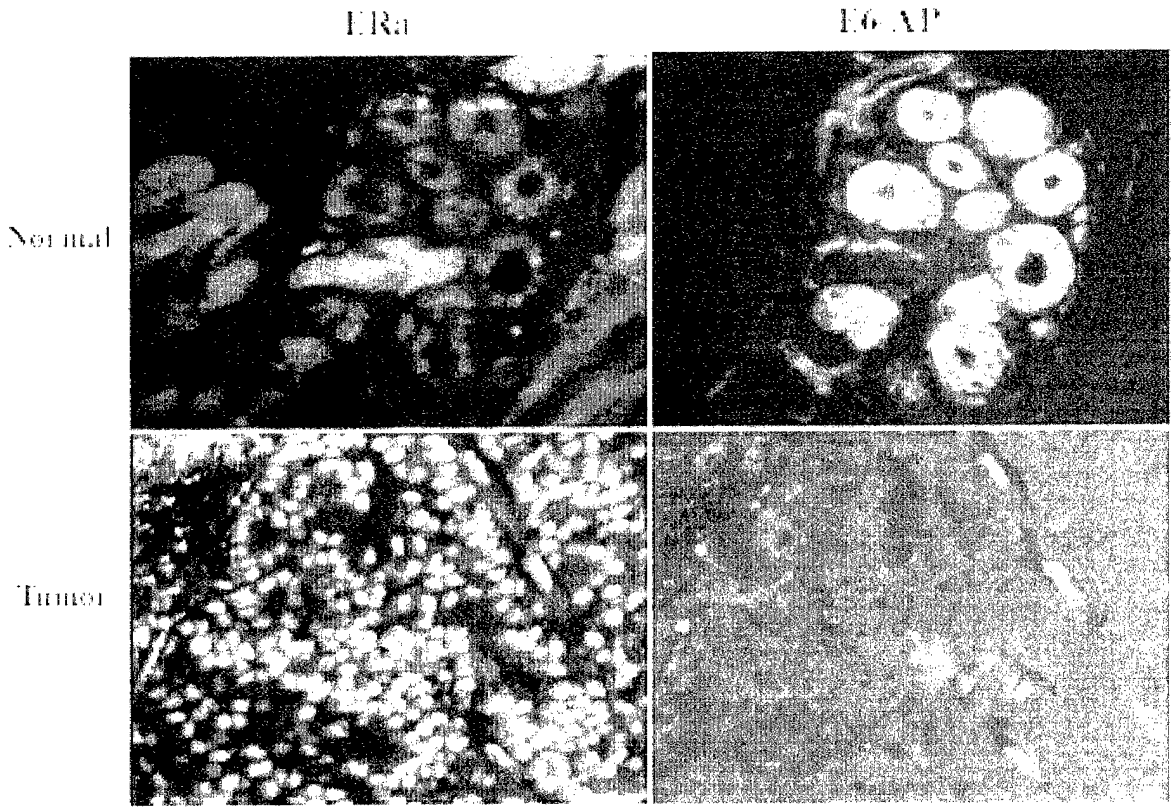


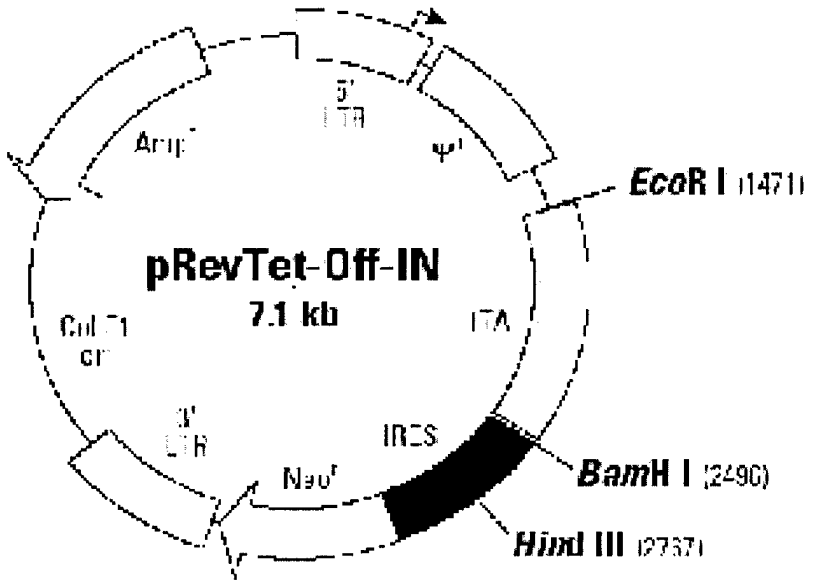
Figure 9. The expression of E6-AP is inversely correlated with that of ERα. The expression patterns of E6-AP and ERα in breast tumors and normal tissues were studied by means of dual immunofluorescent staining using antibodies against E6-AP and ER alpha. This picture is a typical example from the 8 pairs of samples studied. E6-AP is seen as red spot, while ERα is seen as green spot. In the normal breast tissues, ERα is expressed in the nuclei of epithelial cells in a concordant manner, whereas E6-AP is highly and broadly expressed in the epithelial cells mostly in the cytoplasm. In comparison with its normal controls, the expression level of E6-AP is lower in tumors, while the expression of ERα is higher. Altogether, 5 out of 8 tumors that have lower levels of E6-AP express higher levels of ERα.

#	E6-AP	ERα	#	E6-AP	ERα
1	2	4	11	0	3
2	3	3	12	2	1
3	0.5	3	13	0	3
4	1	2	14	0	0
5	1.5	3	15	2	2
6	1	3	16	2	3.5
7	0	0	17	2	4
8	0.5	3	18	0.5	1
9	1	3	19	0.5	0
10	1	3			

(Spearman Rank Correlation Coefficient r=0.503, $p<0.05$)

Figure 10. Correlation of the expression of E6-AP with that of ER-α in breast tumors. Expression levels of E6-AP and ERα from fluorescent immunohisto-chemical analysis were artificially graded according to the intensity of the respective colors; red for E6-AP and green for ERα. ERα is expressed in the nucleus, whereas E6-AP is expressed mostly in the cytoplasm. "0" represents negative expression and "0.5" represents very low expression. From "1" to "4" represent the gradually increasing levels of expression from low to high. Spearman Rank Correlation Coefficient for the expression of E6-AP with that of ERα is 0.503, $p<0.05$.

A.



B.

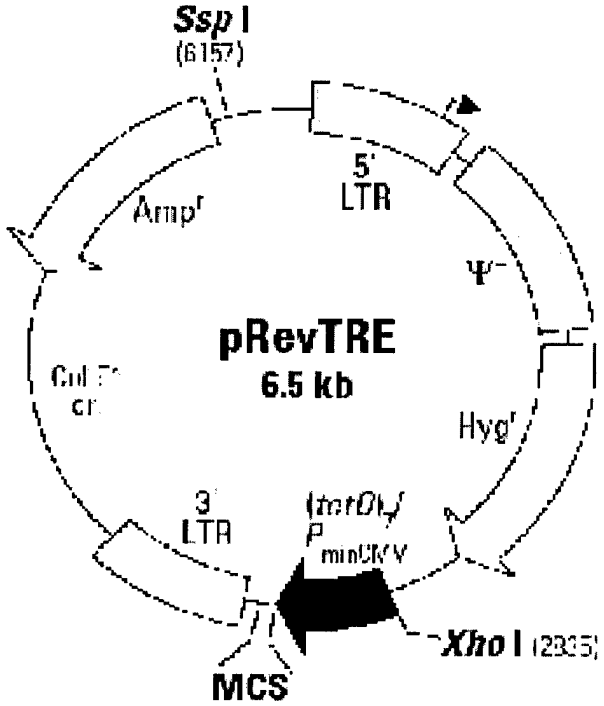


Figure 11. Schemastic structures of pRevTet-Off-In vector and pRevTRE vector. A. pRevTet-Off-In; B. pRevTRE.

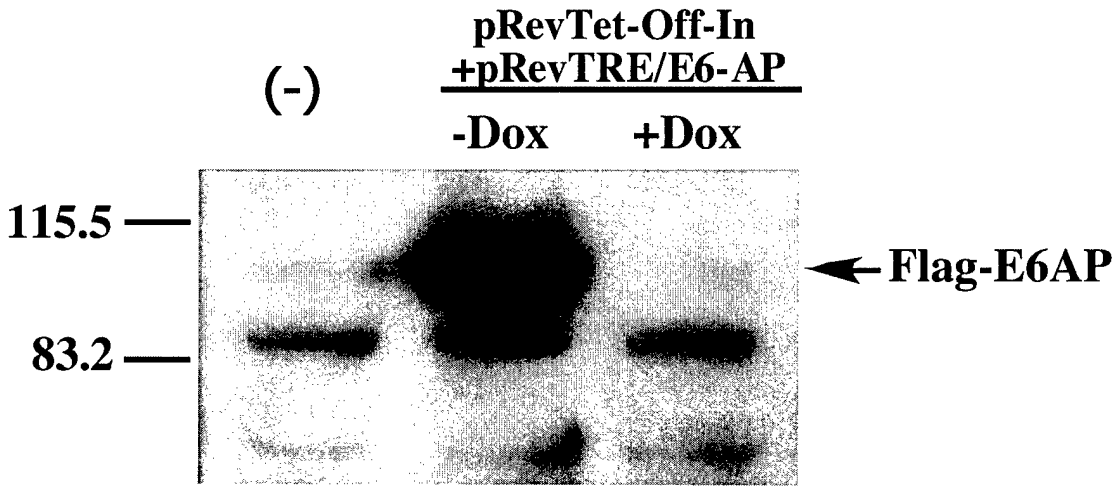


Figure 12. Cotranfection of pRevTet-Off-In and pRevTRE/E6AP plasmids into Hela cells. Hela cells were maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-four hours before transfection, $1x10^6$cells were plated in three 10 cm Falcon dishes. Cells in two of the three dishes were transfected with 5 μg of pRevTet-Off-In and pRevTRE/E6AP-WT by using Fugene 6 transfection reagent (Roche). The third dish of cells were untransfected and used as a control. Dox was added to one of the two transfected dishes 4 hrs later. After 24 hr, cells were harvested and lysed and equal amounts of proteins were resolved by a 10% SDS-PAGE then transfered to nitrocellulose membrane. The expression of recombinant E6-AP was detected using an anti-Flag antibody. a. (-), untransfected Hela cells, b. Hela cells transfected with pRevTet-Off-In and pRevTRE/E6AP and no Dox was added to the culture. c. Hela cells transfected with the same plasmids but Dox was added to the culture.

Appendix 2

Article published in *Molecular Cancer*.

Molecular Cancer

BioMed Central

Review

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The roles of sex steroid receptor coregulators in cancer

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Abstract

Sex steroid hormones, estrogen, progesterone and androgen, play pivotal roles in sex differentiation and development, and in reproductive functions and sexual behavior. Studies have shown that sex steroid hormones are the key regulators in the development and progression of endocrine-related cancers, especially the cancers of the reproductive tissues. The actions of estrogen, progesterone and androgen are mediated through their cognate intracellular receptor proteins, the estrogen receptors (ER), the progesterone receptors (PR) and the androgen receptor (AR), respectively. These receptors are members of the nuclear receptor (NR) superfamily, which function as transcription factors that regulate their target gene expression. Proper functioning of these steroid receptors maintains the normal responsiveness of the target tissues to the stimulations of the steroid hormones. This permits the normal development and function of reproductive tissues. It can be inferred that factors influencing the expression or function of steroid receptors will interfere with the normal development and function of the target tissues, and may induce pathological conditions, including cancers. In addition to the direct contact with the basal transcription machinery, nuclear receptors enhance or suppress transcription by recruiting an array of coactivators and corepressors, collectively named coregulators. Therefore, the mutation or aberrant expression of sex steroid receptor coregulators will affect the normal function of the sex steroid receptors and hence may participate in the development and progression of the cancers.

Introduction

The mammary gland, the ovary and the uterus in females, and the testis and the prostate gland in males are the main target tissues of sex steroid hormones including estrogen, progesterone, and androgen. Estrogen is important for the growth, differentiation and function of both female and male reproductive tissues [1,2], whereas progesterone is an essential regulator of the reproductive events associated with the establishment and maintenance of pregnancy, including ovulation, uterine and mammary gland development [3]. Androgen is involved in the development and physiological function of male accessory sex organs [4], and it is also indispensable for the normal development and function of female reproductive tissues. These hormones exert their functions in the target tissues through their specific intracellular receptors, the estrogen receptors (ER), the progesterone receptors (PR) and the androgen receptor (AR), which belong to the nuclear re-

ceptor (NR) superfamily and function as transcription factors to regulate the target gene expression [5]. The abnormal expression or function of these receptors has been implicated in tumors of reproductive organs in both genders. Furthermore, the development of resistance to hormonal replacement therapy for either breast or prostate cancers is also related to aberrant expression, mutation of the genes and abnormal functioning of the respective steroid receptors.

As members of the NR superfamily, the sex steroid receptors, ER, PR and AR, share the characteristic structure with other nuclear receptors (NRs): an amino-terminal activation, AF-1 (A/B domain); the DNA-binding domain (DBD) (C); a hinge region (D domain); and a carboxy-terminal ligand-binding domain (LBD) (E), which contains a second activation function, AF-2 [5]. In the absence of hormones, NR is sequestered in a non-productive form associated with heat shock proteins and other cellular chaperones. In this state, NR is inactive and unable to influence the transcription rate of its target gene promoters [5,6]. Upon binding with the cognate hormones, the receptors undergo a series of events, including conformational changes, dissociation from heat shock protein complexes, dimerization, phosphorylation, and nuclear translocation, which enables their binding to hormone-response elements (HREs) within the regulatory regions of target genes [5,6]. The binding of hormones to the HREs causes the recruitment of coactivators and basal transcription machinery, leading to the upregulation of target gene transcription.

Coactivators are factors that can interact with NRs in a ligand-dependent manner and enhance their transcriptional activity. Corepressors are factors that interact with NRs, either in the absence of hormone or in the presence of antihormone, and repress their transcriptional activity. Both types of coregulators are required for efficient modulation of target gene transcription by steroid hormones. Therefore, changes in the expression level and pattern of steroid receptor coactivators or corepressors, or mutations of their functional domains can affect the transcriptional activity of the steroid hormones and hence cause disorders of their target tissues. This review will summarize our current understanding about the roles that the coactivators and corepressors may play in the development and progression of cancers in both male and female reproductive tissues.

The SRC family

The SRC (steroid receptor coactivator) family is composed of three distinct but structurally and functionally related members, which are named SRC-1 (NcoA-1), SRC-2 (TIF2/GRIP1/NcoA-2), and SRC-3 (p/CIP/RAC3/ACTR/AIB1/TRAM-1), respectively [5]. Sequence analysis of SRC proteins identified a basic helix-loop-helix (bHLH) domain and two Per-Arnt-Sim (PAS) domains in the amino-terminal region, a centrally located receptor-interacting domain (RID) and a C-terminal transcriptional activation domain (AD). The bHLH/PAS domain is highly conserved among the SRC members and it serves as a DNA binding and protein dimerization motif in many transcription factors. Detailed analysis revealed three conserved LXXLL motifs (NR box) in the RID, which appear to contribute to the specificity of coactivator-receptor interaction. Histone acetyltransferase (HAT) activity was identified in the C-terminal region of SRC members and there also exist activation domains that can interact with the CREB-binding protein (CBP). The members of the SRC family interact with steroid receptors, ER, PR and AR, and enhance their transcriptional activation in a ligand-dependent manner [5].

SRC-1 was the first coactivator for the steroid receptor superfamily that was cloned and characterized [7]. SRC-1 is a common transcription mediator for nuclear receptors, functioning through its HAT activity and multiple interactions with agonist-bound receptors. SRC-1 exhibits a broad range of specificity in the coactivation of the hormone-dependent transactivation of nuclear receptors, including PR, ER, GR (glucocorticoid receptor), TR (thyroid hormone receptor), and AR. Targeted deletion of SRC-1 gene in mice has indicated that SRC-1 is required for efficient steroid hormone action in vivo; for estrogen and progesterone action in the uterus and mammary gland, and for androgen action in the prostate and testis [8].

The role of SRC-1 in the development or progression of cancers is not clear. Although it is an important coactivator for ER and PR, there have been no positive results showing that the expression of SRC-1 is altered in breast cancers or ovarian cancers. However, results from different groups indicated that SRC-1 is involved in the progression of prostate cancers. Using RT-PCR (reverse transcriptase-polymerase chain reaction), Fujimoto and colleagues found that the expression levels of SRC-1 were higher in higher grade prostate cancers or cancers with a poor response to endocrine therapy [9]. At the same time, Gregory *et al* reported that SRC-1 expression was elevated, together with the expression of AR, in recurrent prostate cancers [10]. Gregory *et al* found that SRC-2 is also overexpressed in recurrent prostate cancers. Overexpression of SRC-1 and SRC-2 confers on AR an increased sensitivity to the growth-stimulating effects of low androgen concentrations. This change may contribute to prostate cancer recurrence after androgen deprivation therapy.

SRC-3 is the most distinct among the three members of SRC family; it coactivates not only the nuclear receptors but also other unrelated transcription factors such as

those in the cAMP or cytokine pathways. Compared with the widespread expression of SRC-1 and SRC-2, expression of SRC-3 is restricted to few tissues, including the uterus, the mammary gland and the testis [11]. Disruption of SRC-3 gene in mice causes severe growth and reproductive defects, such as the retardation of mammary gland development [12]. Amplification and overexpression of SRC-3 in human breast and ovarian cancers have been observed [13–17]. Bautista *et al* reported that the AIB1 (SRC-3) amplification/overexpression was correlated with ER and PR positivity [14]. However, Bouras *et al* found that SRC-3 had an inverse correlation with steroid receptors, but a positive correlation with HER-2/Neu and p53 expression [17]. Despite of the conflicting results, the overexpression of SRC-3 in breast and ovarian tumors indicates that SRC-3 is an important factor in the tumorigenesis of the mammary gland and ovary. There is no clear evidence about the possible roles of SRC-3 in prostate tumor development and progression.

SRA/SRAP

The steroid receptor RNA activator (SRA) is a unique coactivator for steroid receptors, PR, ER, GR, and AR. Differing from the other coactivators, SRA was found to function as a RNA transcript instead of as a protein [18]. Besides, SRA existed in a ribonucleoprotein complex containing SRC-1 and it mediated transactivation through the AF-1 domain located at the N-terminal region of nuclear receptors, distinguishing it from the other coactivators [18].

SRA is expressed in normal and malignant human mammary tissues [15,19]. Compared with the adjacent normal region, elevated expression of SRA was found in breast tumors [15]. Although it is currently unknown whether the expression of SRA is correlated with that of PR or ER, the increase in the SRA levels in tumor cells may contribute to the altered ER/PR action, which is known to occur during breast tumorigenesis.

Recently, Kawashima *et al* reported the cloning and characterization of a novel steroid receptor coactivator from a rat prostate library [20]. The nucleotide sequence of this coactivator has 78.2% identity to that of human SRA, however, the cDNA of this coactivator can be transcribed into a functional protein and exerts its coactivation function as a protein instead of an RNA transcript [20]. Therefore, it was designated as steroid receptor activator protein, SRAP. Kawashima *et al* demonstrated that SRAP could enhance the transactivation activity of AR and GR in a ligand-dependent manner. The mRNA of SRAP was expressed in all the rat prostate cancer cell lines examined, while that of SRA was expressed in all the human prostate cancer cell lines. The expression level of SRA is higher in androgen-independent PC-3 cells compared with that of the androgen-dependent cell lines, DU-145 and LNCaP. Taken together, these results suggested that both SRA and SRAP play an important role in NR-mediated transcription in prostate cancer.

E6-AP/RPF1

E6-associated protein, E6-AP, and RPF1, the human homolog of yeast RSP5, are E3 ubiquitin-protein ligases that target proteins for degradation by the ubiquitin pathway. They are also characterized as coactivators of steroid receptors. It has been demonstrated by transient transfection assay that RPF1 and E6AP can potentiate the ligand-dependent transcriptional activity of PR, ER, AR, GR, and other NRs [21,22]. Furthermore, they also act synergistically to enhance the transactivation of NRs [22]. Additionally, the coactivation functions of E6-AP and RPF1 are not dependent on the E3 ubiquitin-protein ligase activity.

E6-AP is expressed in many tissues including the uterus, ovary, testis, prostate and mammary gland. It is important in the development and function of these tissues, since E6-AP null mutant mice exhibited defects in reproduction in both male and female mice [23].

The first evidence of a relationship between E6-AP and cancer was obtained from the study of a spontaneous mouse mammary tumorigenesis model. In this spontaneous model, E6-AP was overexpressed in mammary tumors when compared with normal tissues [24]. Recently, we examined the expression pattern of E6-AP in biopsy samples of human breast cancers. Our results showed that E6-AP expression was decreased in tumors in comparison to the adjacent normal tissues (Gao *et al*, unpublished data). In addition, the expression of E6-AP was inversely correlated with that of ER in breast tumors, and the decreased expression of E6-AP was stage-dependent. Interestingly, the decreased expression of E6-AP was also found in human prostate cancers (Gao *et al*, unpublished data). ER plays a major role in breast cancer development, and PR is also a target of estrogen. Thus, changes in the expression level of E6-AP, a coactivator for ER and PR, might interfere with the normal functioning of ER and PR, hence participating in the formation and progression of breast tumors. In a similar way, the altered expression of E6-AP might influence the normal functioning of AR, which plays a major role in the progression of prostate cancers.

ASC-2/TRBP/AIB3

ASC-2 (the nuclear protein-activating signal cointegrator-2), also called AIB3 (the amplified in breast cancer 3) and TRBP (TR-binding protein), has recently been characterized as a NR coactivator [25]. ASC-2 interacted with NRs, such as retinoid acid receptor (RAR), TR, ER, and GR, and stimulated the ligand-dependent and AF2-dependent transactivation of the NRs either alone or in conjunction with CREB-binding protein (CBP)/p300 and SRC-1. Sub-

sequent study showed that ASC-2 also interacted with SRF (the serum response factor), AP-1 (the activating protein-1), NF-κB (the nuclear factor-κB), and potentiated transactivation by these mitogenic transcription factors [26]. This suggests that ASC-2 is a multifunctional transcription integrator molecule.

ASC-2 is likely involved in the tumorigenesis of mammary gland, because it is amplified and overexpressed in human breast cancer specimens as well as in all the human breast cancer cell lines examined. Moreover, it may also regulate cellular proliferation or tumorigenesis by the direct interaction with SRF, AP-1 and NFκB.

L7/SPA

L7/SPA, L7/switch protein for antagonists, is a 27 kDa protein containing a basic leucine zipper domain. L7/SPA is an antagonist specific transcriptional coactivator because it can only potentiate the partial agonist activity of some antagonists, including tamoxifen and RU486, but has no effect on the agonist-mediated transcription [27]. The study by Graham *et al* indicated that the relative levels of the coactivator, L7/SPA, vs. the corepressors, which suppress the partial agonist activity of tamoxifen or RU486, might determine whether the agonist or antagonist effects of these mixed antagonists predominate in a tissue or tumor [28]. This unique property of L7/SPA could partially explain the development of resistance to hormone therapy for breast cancers.

ARAs

ARAs, androgen receptor-associated proteins, is a group of factors that can bind to AR and modulate its transcriptional activity. Based on their molecular weights, these factors were named ARA70, ARA160, ARA54, ARA55, ARA267 and ARA24.

ARA70, which has a molecular weight of 70-kDa, is also named as RFG (RET fused gene) and ELE1. ARA70 was first described as an AR-specific coactivator by Chang's group in 1996 [29]. In that report, ARA70 was demonstrated as a factor, which specifically interacts with AR and enhances the transcriptional activity of AR in response to the stimulation of androgens, including testosterone and dihydrotestosterone, but not the antiandrogen, hydroflutamide (HF). Later, it was reported that ARA70 could also interact with and facilitate the agonist activity of antiandrogens, including cyproterone (CPA), HF, and bicalutamide (casodex) [30]. Recent studies by other groups showed that ARA70 was not a specific coactivator for AR; it could also interact with PR or GR [31,32]. However, studies on the expression patterns of ARA70 in different cell lines and human cancer samples showed that the expression of ARA70 was decreased in prostate cancer [31,33–35] and breast cancer, [36] while it was increased in ovarian cancers. In breast, loss of ARA70 protein expression was found in 60% of HER2 positive breast cancers, while only 33% of HER2 negative breast cancer samples lost the expression [36]. Since androgen plays an inhibitory role for breast cancer cell growth, and HER2 stimulates the growth of breast cancers, loss of the expression of AR and/or ARA70 in breast might confer a growth advantage to these cells. In prostate, ARA70 mRNA is highly expressed in the normal epithelial cells, while benign prostatic hyperplasic and cancer cell lines express either lower or no ARA70 [36]. Methylation might be responsible for the lack of expression of ARA70 in some prostate cancer cells such as DU145 [36]. The expression of ARA70 in prostate cancer cells seems to be regulated by both ER and AR, since the prostate cancer cell line, PC-3, responded to estrogen/androgen and their respective antagonists differently in the parental PC-3 cells (AR-negative) and its derived AR-positive cells.

Other members of the ARA group, such as ARA54, ARA55, ARA24, ARA160, and ARA267 were also implicated in prostate tumors [35,37–40]. The expression of these coactivators was more or less altered in human prostate cancer cell lines or biopsy samples. However, the exact roles of these factors in prostate tumorigenesis need to be determined.

The PIAS family

The PIAS (protein inhibitor of activated signal transducer and activator of transcription) family is composed of a group of proteins that share a high sequence homology [41]. The first member of this family, PIAS1, was characterized as a coactivator for AR [42]. Through its N-terminal LXXLL motifs, PIAS1 interacted with and coactivated the AR transcriptional activity in a ligand-dependent manner [42]. Besides, PIAS1 could also modulate the activities of steroid receptors such as GR, PR and ER [42,43]. PIAS1 was expressed predominantly in the testis [42]. Furthermore, overexpression of PIAS1 was found in 33% of the prostate cancer samples examined [35]. These data suggested a possible role that PIAS1 may play in normal or cancer development of the testis or prostate.

Another important member of the PIAS family is called PIASxα or ARIP3 (AR-interacting protein 3). PIASxα/ARIP3 is similar to PIAS1 in that it is also expressed predominantly in the testis, and functions as a coactivator for AR [43,44].

SNURF

The small nuclear RING finger protein, SNURF, was identified in a yeast two-hybrid screening using the DBD of AR as a bait [45]. SNURF interacted with AR, GR and PR, and enhanced their transcriptional activity in a ligand-dependent fashion. It also potentiated the basal transcrip-

tion from steroid-regulated promoters [45]. SNURF is a nuclear protein. The expression of SNURF was relatively high in the brain, but low in the testis, prostate, seminal vesicles, spleen and kidney [45]. Moreover, the nuclear localization signal (NLS) in SNURF was found to be able to facilitate the nuclear import and export of AR [46], which is important for normal functioning of AR transactivation.

BRCA1

BRCA1 is a breast cancer susceptibility gene, and its inherited mutations are correlated with an increased risk of breast and ovarian cancers [47]. The role of BRCA1 in cancer development is quite complex. On one hand, BRCA1 was shown to coactivate p53, modulate p300/CBP expression, and function as a ligand-independent corepressor for ER, PR, and AR [48–50]; on the other hand, it was shown that it could enhance the ligand-dependent AR transactivation in both breast and prostate cancer cell lines, especially in the presence of exogenous SRC family members [51]. These results are somewhat controversial regarding the influence of BRCA1 on AR activity. ER and PR play key roles in breast cancer development and progression, and AR signaling in the breast has protective effect. Thus, it is reasonable to speculate that the normal expression of BRCA1 probably protect the breast from tumorigenesis by suppressing the ER and PR signaling pathway and promoting the AR activity. Mutation of the BRCA1 gene, therefore, increases the risk of developing cancer.

In a recent study by Ko *et al.* a genomic transcript, GT198, that mapped to the human breast cancer susceptibility locus (17q12-q21), was characterized as a coactivator for nuclear receptors such as AR, ER, PR, GR, etc [52]. GT198 has a tissue-specific expression pattern; it is expressed highly in testis, moderately in thymus, spleen, and pituitary, and hardly detected in other tissues. The role of this novel coactivator in cancers of testis or breast needs to be explored.

CBP/p300

CREB-binding protein (CBP) was initially characterized as a coactivator required for efficient transactivation of cAMP-response element-binding protein. p300 was first identified as a coactivator of the adenovirus E1A oncoprotein. CBP and p300 share many functional properties. Both of them function as coactivators for multiple NRs as well as p53 and NF-kB; both possess intrinsic HAT activity and both can recruit HAT and p/CAF (CBP/p300-associated factor) [5]. Besides, CBP/p300 interacts with members of SRC family and synergizes with SRC-1 in the transactivation of ER and PR [53]. Based on its wide expression and multiple functions, it is speculated that CBP/p300 might participate in the process of tumor initiation and progression.

N-CoR/SMRT

N-CoR and SMRT are both corepressors of numerous transcription factors, including steroid hormone receptors. Both N-CoR and SMRT interact with the nuclear receptors through the RIDs located in the C-terminal portion of the proteins, while their transcriptional repression domains were mapped to the N-termini [54]. N-CoR/SMRT also associates with HDAC3 (histone deacetylase 3) in large protein complexes, which is an important pathway for transcriptional repression. Corepressors N-CoR and SMRT interact with the NRs either in the absence of agonists (in the case of TR and RAR), or in the presence of antagonists (in the case of steroid receptors) [54]. As mentioned above, corepressors, N-CoR and SMRT, can suppress the partial agonist activity of antagonists, counteracting the effects of L7/SPA. The alteration of the expression of these corepressors changes the balance of corepressors to coactivators that are bound to the transcription complex via the antagonist-occupied steroid receptors. This might determine whether the outcome is inhibitory or stimulatory, and therefore determine whether tamoxifen-resistance will occur or not.

Other coregulators

In addition to the above-mentioned coactivators and corepressors, there are many other factors that have been characterized as sex steroid receptor coregulators. These include HMG-1/2 (the chromatin high-mobility group protein-1, 2), TIP60 (Tat-interacting protein), PNRC1/2 (proline-rich nuclear receptor coregulatory protein-1, 2), Cdc25B, Uba3 (ubiquitin-activating enzyme 3), and RTA (repressor of tamoxifen transcriptional activity) [55–60]. At present, it is not clear whether these coregulators are involved in the development of cancers.

Conclusion

Steroid receptors activate their target gene transcription in response to the hormonal stimulus. Their transactivation activities are modulated by coregulators (coactivators and corepressors). Different coregulators exert their actions through different mechanisms. Involvement of coregulators in the development and progression of cancers is complex. Most of the steroid receptor coactivators and corepressors identified so far are widely expressed. They usually can modulate the transactivation of multiple receptors. On the other hand, the transactivation function of a single nuclear receptor in certain tissues is usually regulated by multiple coregulators. Much evidence supports the importance of coregulators in tumorigensis and the development of hormone-resistance in breast or prostate cancers. The understanding of the mechanisms of the actions of these coregulators will be helpful for the development of new cancer therapies.

Authors' contributions

XG and BWL drafted the manuscript and ZN supervised and performed the final editing. All authors read and approved the final manuscript.

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Appendix 3

Article submitted to *Cancer Research*.

Title Page

A. Full Title:

E6-associated protein, E6-AP is involved in the carcinogenesis of human mammary and prostate glands

B. Running Title:

Role of E6-AP in the carcinogenesis of breast and prostate

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Key Words:

Steroid Receptors, Coactivators, Ubiquitin-Proteasome Pathway, E6-AP, Breast and Prostate Carcinogenesis

Abstract

The E6-associated protein (E6-AP) is a dual function protein. It acts as an E3 ubiquitin-protein ligase as well as steroid hormone receptors (SHRs) coactivator. Considering the influence of SHRs and their coactivators in the normal development and tumorigenesis of reproductive organs of both genders, we studied the roles of E6-AP in the tumorigenesis of breast and prostate tissues. Our immunohistochemical analysis data demonstrated that the expression of E6-AP protein is decreased in human invasive breast and prostate carcinomas compared to their adjacent normal tissues, and this downregulation of E6-AP is accompanied by the upregulation of estrogen receptor-α (ERα) in breast and androgen receptor in prostate carcinomas. Furthermore, our in vitro studies and data from E6-AP knockout mice indicated that E6-AP modulates the expression and function of ERα in mammary glands by promoting its degradation through the ubiquitin-proteasome pathway.

Introduction

Breast and prostate carcinomas are among the top three cancers in American women and men, respectively. It is well established that the steroid hormones, estrogen, progesterone and androgen are the principal regulators of mammary gland and/or prostate gland development and function (1-3). These steroids exert their effect on the target tissues via their cognate intracellular receptors, estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR), all of which are members of the nuclear hormone receptor superfamily and act as transcription factors. The steroids, estrogen,

progesterone and androgen, not only regulate the normal growth and development of their target tissues, but also play important roles in tumorigenesis of these tissues (1-3). The abnormal expressions of ER and AR and mutations within these receptors have been associated with cancers of breast and prostate, respectively. Furthermore, the development of resistance to the antihormone therapies for breast and prostate cancers are also related with aberrant expression and mutations of steroid hormone receptors (SHRs) (1-3). Currently, the detailed molecular mechanisms, by which the SHRs regulate the development and progression of breast cancer and prostate cancers remain largely unknown. Theoretically, any factor that can influence the expression or functions of ER and AR might contribute to the development and progression of breast and prostate cancers. In this study, we explored the role of the dual function protein, E6-associated protein (E6-AP) in the development and progression of breast and prostate carcinomas.

It has been demonstrated that the ubiquitin-proteasome pathway is required for the degradation and proper function of SHRs (4-6). E6-AP is a coactivator of SHRs and an important component of the ubiquitin-proteasome pathway (an E3 ubiquitin-protein ligase) (7, 8). Therefore, we tested whether changes in the expression pattern of this dual function protein may be involved in the carcinogenesis of breast and prostate. Our data indicated that the expression of E6-AP protein is downregulated in advanced-stage carcinomas of breast and prostate. Furthermore, this downregulation is accompanied by upregulation of ERα and AR. We also demonstrated that E6-AP modulates the level of ERα protein in mammary epithelial cells by promoting its degradation via the ubiquitin-proteasome pathway.

Materials and Methods

Human tumor samples. Formalin-fixed, paraffin-embedded samples of 13 invasive breast carcinomas (IBC), 20 ductal in situ carcinoma (DCIS) of breast, and 7 prostate carcinomas were included in this study. The tumor specimens with either internal normal tissue within the same section (IBC and DCIS) or normal tissues taken from normal area adjacent to the tumor of the same patient (prostate cancer) were sectioned and mounted on the same slide. Use of human tissues for this study was approved by the local institutional review boards.

Mouse mammary tissues. The inguinal mammary glands taken from E6-AP-knock-out mice (9) and wild-type control littermate mice were fixed in Formalde-Fresh (Fisher Scientific, Pittsburgh, PA) for 18-24 hrs, embedded in paraffin and sectioned on a microtome.

Immunohistochemistry. Five-μm thick paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded alcohol. Antigen was retrieved by boiling the slides in the antigen unmasking solution (Vector Laboratories, Burlingame, CA) in a microwave oven for 10 min and endogenous peroxidase activity was quenched by 30 min incubation of the sections in 1% hydrogen peroxide at room temperature. The sections were incubated overnight with 10% normal goat serum in Tris-buffered saline (TBS) at 4 ^{0}C to block the nonspecific immunoreactivity. Antibodies against E6-AP (a

rabbit polyclonal antibody, kindly donated by Dr. Norman J. Maitland), ERα (NCL-ER-6F11, a mouse monoclonal antibody, Novocastra, UK) and AR (sc-7305, a mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA), at dilutions of 1:200, 1:100 and 1:100, respectively, were added to the sections at room temperature for 1 to 2 hrs. For the detection of the immunoreactivity, sections were then incubated with biotinylated anti-rabbit (E6-AP) or anti-mouse (ERα and AR) antibodies (Bio-Rad Laboratories, Hercules, CA), washed in TBS, incubated with streptavidin-conjugated peroxidase (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), washed, and developed with 3,3'-diaminobenzidine (DAB substrate kit, Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. Finally, the sections were counterstained with hematoxylin and coverslipped for bright field microscopy after dehydration in graded alcohol and clearance in xylene. Negative controls were included in each experiment by omission of the primary antibody.

Evaluation of immunostained slides. The staining results of E6-AP were evaluated using an automated cellular imaging system (ACIS, Chromavision Medical Systems, Inc., San Juan Capistrano, CA). This system combines color based imaging technology with automated microscopy to provide quantitative information on staining and it measures intensity on a scale of 0 to 256. Since the expression of E6-AP is ubiquitous in breast and prostate epithelial cells, intensity only was used to indicate the expression levels of E6-AP protein. For ERα and AR, which are heterogeneously expressed in breast and prostate epithelial cells, we used H score, which is a combination of the intensity and the percentage of positive cells.

***In Vitro* Expression of ER.** *In vitro* synthesis of radiolabeled human ERα was performed using in vitro transcription and translation kit according to the manufacturer's recommended condition (Promega, Madison, WI).

Protein Degradation and Ubiquitination Assay. The ^{35}S-labeled human ER α was incubated either with or without E6-AP protein purified from *E. coli* and in the presence or absence of the proteasome inhibitor, lactacystin, in a mixture containing 20 mM Tris-HCl (pH7.5), 50mM NaCl, 4mM ATP, 10mM $MgCl_2$, 0.2 mM dithiothreitol, and 4μg of ubiquitin (Sigma) for 1 h at 30^{0}C. Reactions were terminated by boiling samples in the presence of SDS-loading buffer (100 mM Tris-HCl (pH8.0), 200mM dithiothreitol, 4% SDS, 20% glycerol, and 0.2% bromophenol blue). The reaction mixtures were resolved by 10% SDS-PAGE and radiolabeled bands were visualized by autoradiography.

Statistical Methods. Differences between tumor samples and their matched normal tissues were tested using the *Student paired t-test.*

Results and Discussion

To investigate the expression profile of E6-AP in human breast cancer, we compared immunohistochemical expression of E6-AP protein in neoplastic cells of DCIS and IBC to the adjacent normal mammary gland. Figure 1A shows the immunostaining of E6-AP in the normal breast tissue, DCIS and IBC, which were found in the same section of a

breast carcinoma patient. In the normal breast tissue, E6-AP is strongly and ubiquitously expressed in epithelial cells, predominantly in the luminal epithelial cells. When compared with the normal tissue, the immunostaining of E6-AP is greatly decreased in the IBC, whereas there was no significant change of E6-AP expression in the DCIS. A total of 13 IBC cases were immunochemically analyzed for their expression of E6-AP, each of which had their adjacent normal tissue as a control. The immunostaining intensity of the 13 cases was evaluated using an ACIS and the result is shown in Figure 1B. 100% of the samples exhibited decreased immunostaining in IBC compared with the normal glands. On average, there was a 25% reduction in the intensity of the immunostaining in tumors than in the adjacent normal tissues. Student paired *t-test* indicated that the differences were statistically significant (p=0.00001). However, in DCIS, which represents a precursor to IBC, decreased expression of E6-AP was not observed (data not shown), suggesting that the down-regulation of E6-AP protein level is a relatively late event associated with invasive phenotype.

Since E6-AP is involved in the coactivation function of ERα, and furthermore, ERα is a target of the ubiquitin-proteasome pathway, we analyzed the expression of ERα in the IBC and DCIS samples and compared ERα expression pattern with that of E6-AP. As shown in Figure 2, the downregulation of E6-AP protein in IBC was accompanied by the increased expression of ERα in the epithelial cells; whereas in DCIS, high level expression of E6-AP was accompanied by low level expression of ERα protein. These data suggested an inverse correlation between the expression of E6-AP and ERα.

It is known that the normal development and function of mammary gland largely depend on the normal expression and function of ER and other SHRs (10, 11). Furthermore, ER also acts as an important mitogenic factor for breast epithelial cells, indicating that ER plays significant roles in the development and progression of breast cancer (1). The finding of an inverse correlation between the protein levels of ERα and E6-AP raises the possibility that E6-AP might be a factor that regulates the expression levels of ERα in mammary gland and contributes to mammary tumorigenesis. Since E6-AP is an E3 ubiquitin-protein ligase enzyme (8), we hypothesized that E6-AP may promote ER degradation via the ubiquitin-proteasome pathway and if so, loss of E6-AP function may provide growth advantage to the tumor cells by increasing ERα protein levels.

The ubiquitin-proteasome pathway accounts for the selective degradation of short-lived regulatory proteins including many nuclear hormone receptors. Three classes of enzymes are involved in protein ubiquitination, i.e. the E1 ubiquitin-activating enzyme (UBA), E2 ubiquitin-conjugating enzymes (UBCs) and E3 ubiquitin-protein ligases. The UBA activates ubiquitin and transfers it to one of the several UBCs. Then the ubiquitin is transferred from E2 to the target protein, either directly or through an E3 ubiquitin-protein ligase intermediate (12, 13). UBA and UBCs are necessary for the degradation and turnover of ER protein levels (4, 5, 14), which permits continuous responses to changes in the concentration of estrogen. As a rate-limiting factor in the ubiquitin-proteasome pathway, it is possible that E6-AP also plays an important role in the regulation of ER protein levels. To test this hypothesis, we performed *in vitro* protein degradation and ubiquitination assay. ^{35}S-labeled ERα protein was synthesized in vitro by

using an in vitro transcription and translation kit. The ^{35}S-labeled ERα protein was then incubated with ATP and ubiquitin either in the presence or absence of bacterially expressed E6-AP. As expected, E6-AP promoted the degradation of ERα (Fig. 3A), and the proteasome inhibitor, lactacystin, inhibited ERα degradation. To further confirm that E6-AP modulates the levels of ERα , we also examined the ERα protein levels in the mammary glands of E6-AP knockout mice. E6-AP knockout mice exhibited an increased expression of ERα in the mammary epithelial cells as compared to their wild-type litermates (Fig. 3B). These results demonstrated that E6-AP is required for the degradation of ERα through the ubiquitin-proteasome pathway.

Recently, the role of coactivators in tumor development and progression has gained more and more attention. In fact, overexpression of nuclear receptor coactivators, including AIB1 (amplified in breast cancer 1), SRA (steroid receptor RNA activator), and AIB3 (amplified in breast cancer 3), have been implicated in human breast cancers (15-20). It is considered that overexpression of coactivators would upregulate the signaling of steroid receptors, such as ER, which occurs in breast cancer development and progression. On the other hand, decreased expression of a coactivator, ARA70 (androgen receptor-associated protein 70), was also reported in breast and prostate cancers (21), suggesting that the expression levels of coactivators could be either upregulated or downregulated in cancers. Previously, it was published that E6-AP is a coactivator of ER and other steroid receptors. Furthermore, E6-AP is overexpressed in a spontaneous ERα-negative mouse mammary tumor model (22). However, our data suggest that the expression of E6-AP is decreased in invasive breast carcinoma, which express higher levels of ERα, as compared

to the adjacent non-neoplastic epithelium. Since our in vitro data showed that E6-AP is required for the degradation of ERα, we speculate that E6-AP plays important roles in tumorigenesis of mammary gland by modulating ERα protein levels.

Prostate cancer exhibits many similarities to breast cancer in regard to its AR expression and its switch from hormone responsiveness at the earlier stage to the hormone resistance at the later stage (2). The ubiquitin-proteasome pathway is also involved in the modulation of AR transcriptional activity and AR protein levels (6). The changes in the expression of certain AR coactivators have also been associated with prostate cancers (23-26). Therefore, we were interested in knowing whether the expression of E6-AP is also altered in prostate cancers. Similar to that observed in breast cancer, the immunostaining of E6-AP was also decreased in prostate cancers when compared with the matched normal tissues from the same patient. In normal prostate tissues, E6-AP was expressed highly in the luminal epithelial cells, while the expression of E6-AP was decreased in tumor cells (Fig. 4A). The immunostaining of E6-AP of the 7 prostate tumor cases with their normal gland was evaluated using an ACIS. 100% of the tumor samples exhibited a decreased expression of E6-AP compared to the adjacent normal tissues (Fig. 4B). Overall, there was 27% decrease in E6-AP expression in the tumors as compared to the normal tissue and this difference was statistically significant (p=0.000022). An inverse correlation between the expression of E6-AP with that of AR was also seen in prostate cancers (data not shown). These results indicated that E6-AP might participate not only in the carcinogenesis of the breast, but also in the carcinogenesis of other tissues,

such as the prostate. The roles of E6-AP in prostate tumorigenesis may also involve its ubiquitin-proteasome activity.

In conclusion, we present data herein showing that E6-AP is down regulated in breast and prostate tumors and the protein level of E6-AP is inversely associated with that of ER and AR. This study shows that E6-AP plays a role in the carcinogenesis of breast and prostate glands probably through its participation in the degradation of ER and AR proteins via ubiquitin-proteasome pathway.

Acknowledgements

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Figure Legends

Figure 1. Immunohistochemical analysis of E6-AP expression in human breast tissues. A. Representative immunostaining of E6-AP in human breast tissues: a. Nor, normal breast; b. IBC, invasive breast carcinoma; c. DCIS, ductal carcinoma in situ. Enlarged images are shown on the right panel, respectively. B. The immunostaining intensity of E6-AP in both normal breast tissue and IBC of each case, analyzed by ACIS.

Figure 2. Immunohistochemical analysis of E6-AP and ERα in breast carcinomas. Representative immunostaining of E6-AP and ERα in IBC and DCIS: a. ERα in DCIS; b. ERα in IBC; c. E6-AP in DCIS; d. E6-AP in IBC.

Figure 3. Degradation of ERα through the ubiquitin-proteasome pathway. A. In vitro protein degradation and ubiquitination assay: a. Input; b. –E6-AP; c. +E6-AP; d. +E6-AP and lactacystin. B. ERα immunohistochemistry of mammary glands in wild-type and E6-AP knockout mice: a. WT, wild-type mouse mammary gland; b. KO, knockout mouse mammary gland.

Figure 4. Immunohistochemical analysis of E6-AP in human prostate cancers. A. Representative immunostaining of E6-AP in human prostate tissue: a. normal prostate tissue; b. prostate carcinoma. Enlarged images were shown on the right panel. B. Immunostaining intensity of E6-AP in both the normal prostate tissue and tumor tissue of each patient, analyzed by ACIS.

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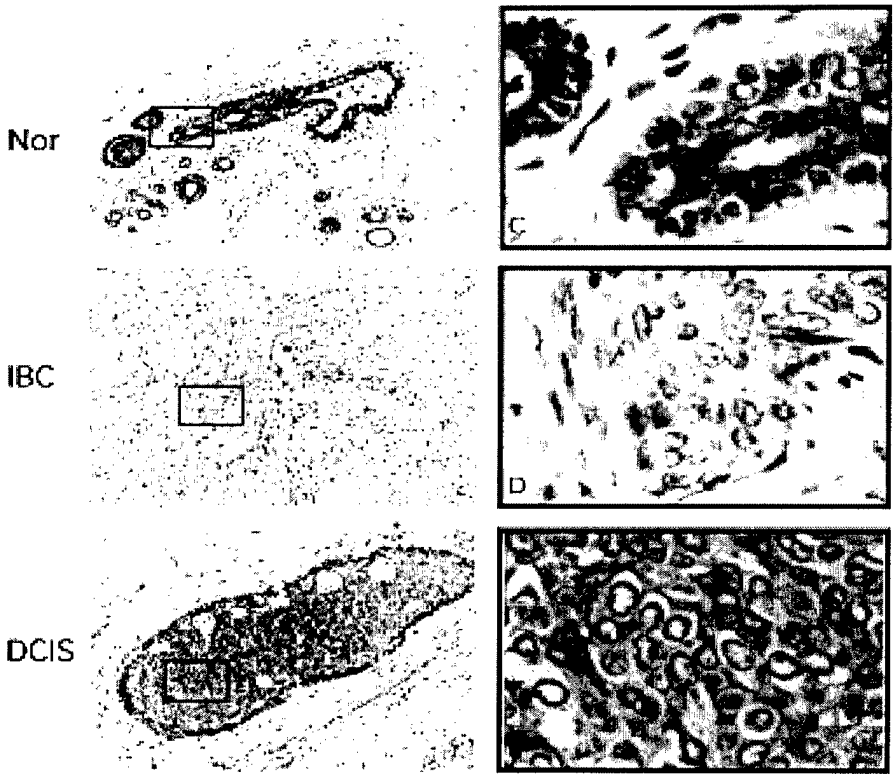
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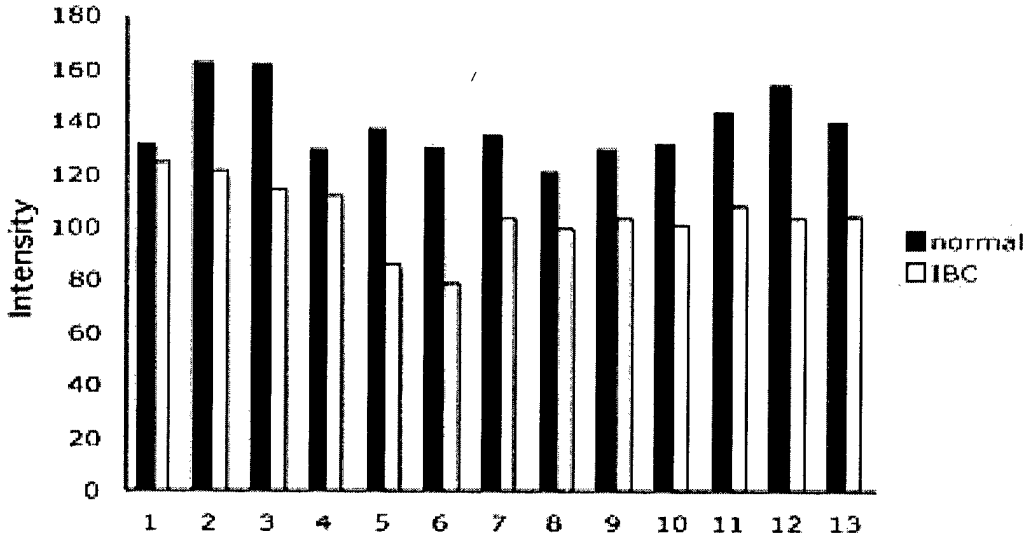
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normal
IBC

Figure 1

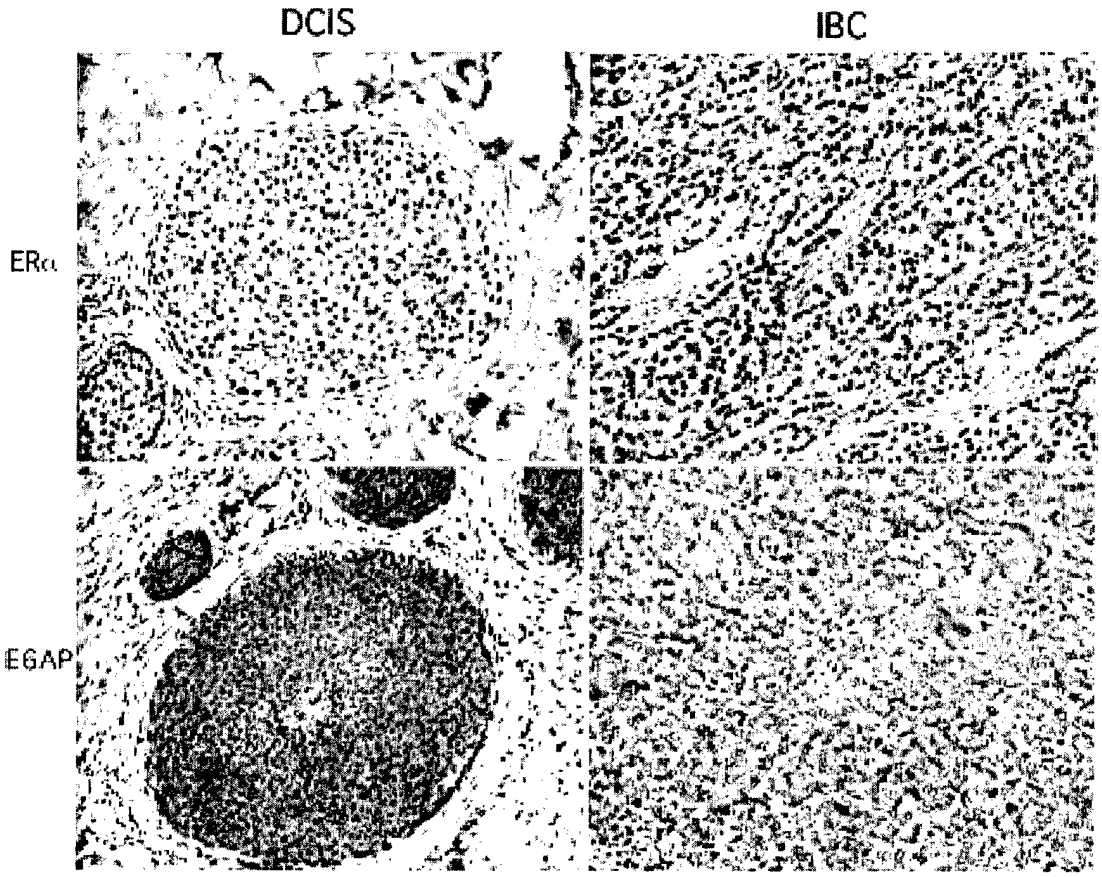
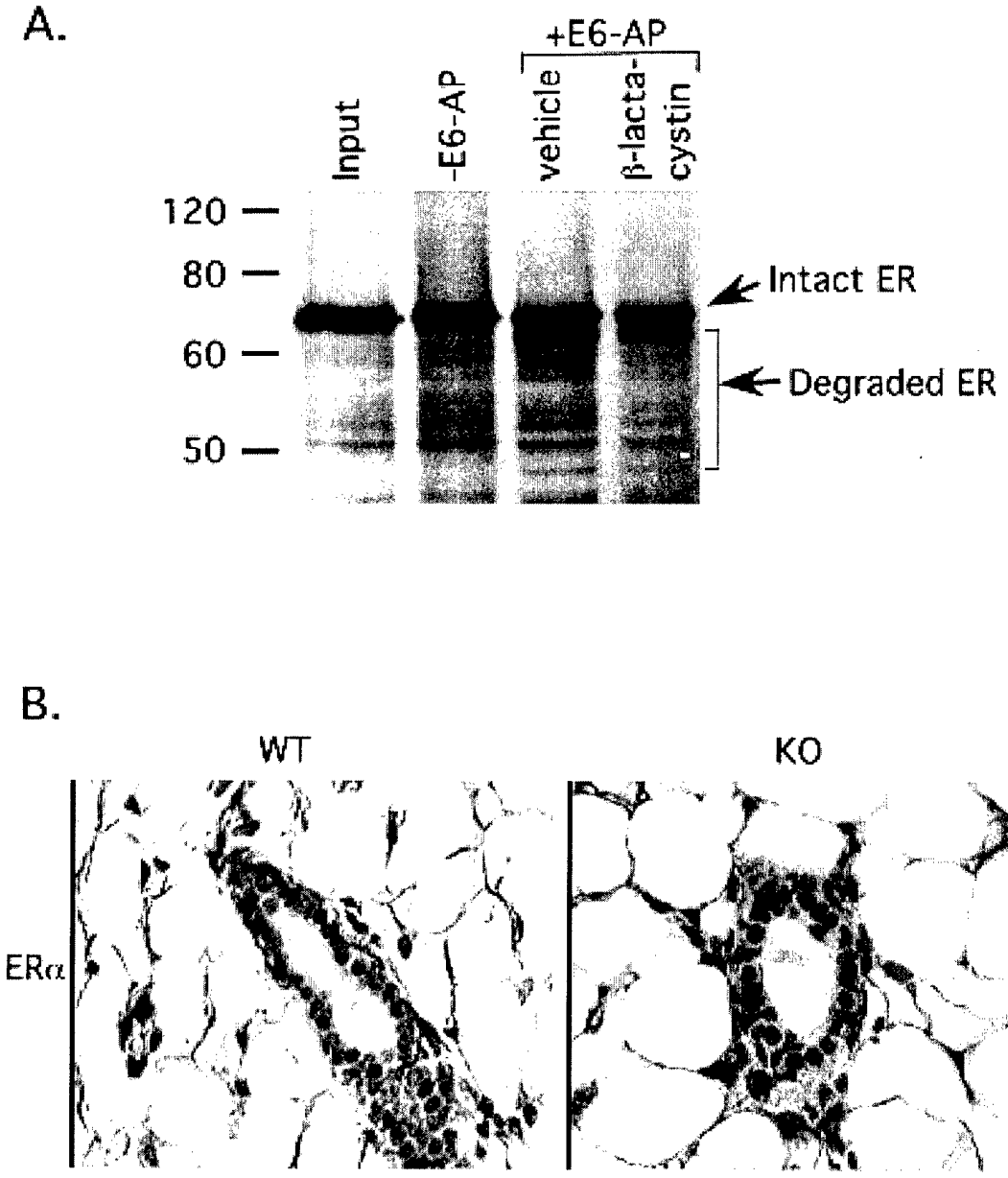


Figure 2


A.
+E6-AP
Input
-E6-AP
vehicle
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80
60
50
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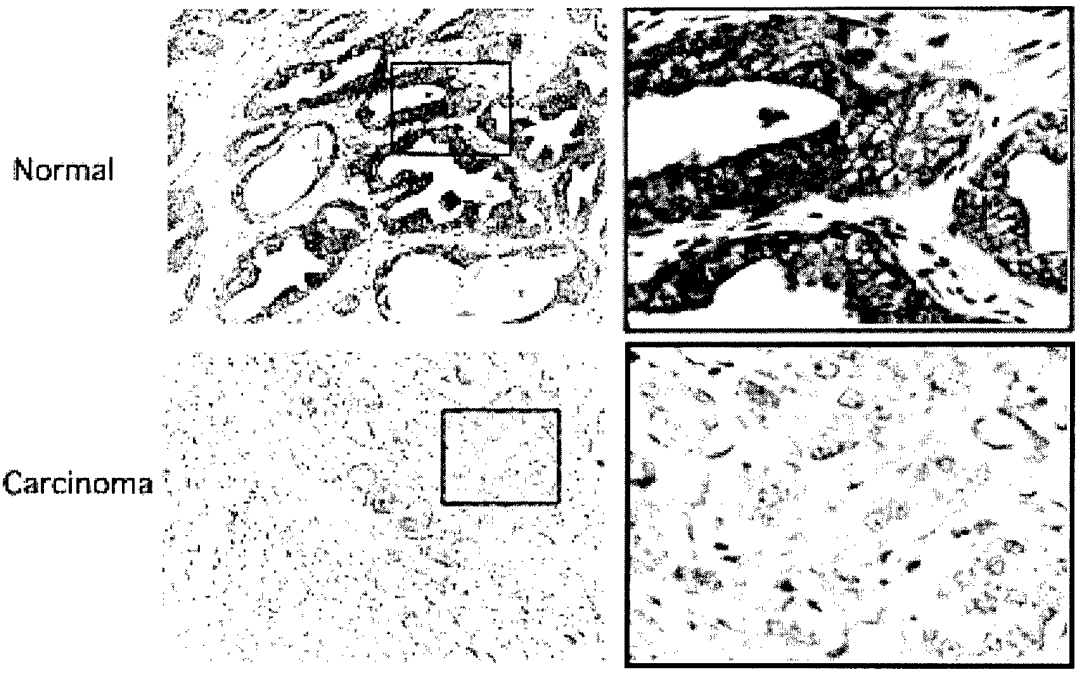
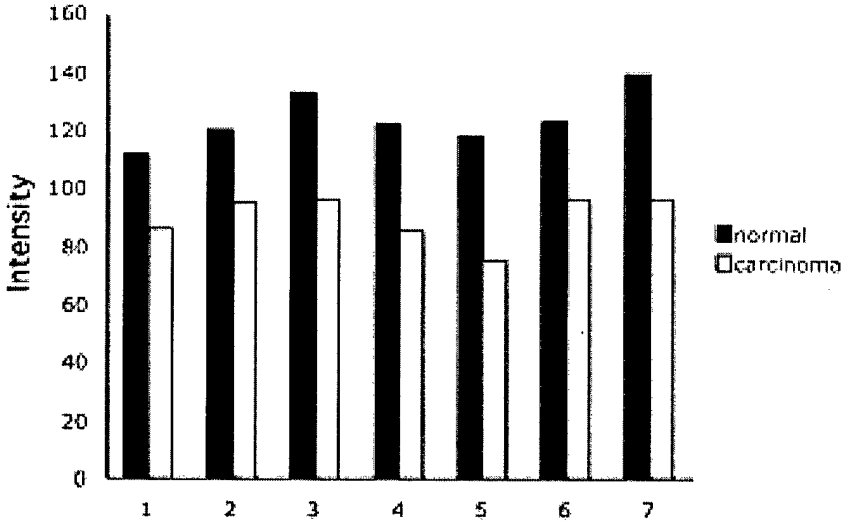
Degraded ER
B.
WT
KO
ERα

Figure 3

A.


Normal
Carcinoma

B.


160
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Intensity
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Figure 4